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THE IMPORTANCE OF UNIFORM CULTURE MEDIA IN THE BACTERIOLOGICAL EXAMINATION OF DISINFECTANTS

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Neither of the two methods for the bacteriological standardization of disinfectants which are in common use today has proven entirely satisfactory. Even in the hands of the most careful workers wide discrepancies in results have occurred, both with the Hygienic Laboratory and Rideal-Walker tests. While it is probably true that a few individuals have succeeded in so controlling their work as to obtain generally uniform and consistent results, a method worthy of general adoption must be capable of yielding satisfactory results in the hands of any well trained bacteriologist. It is hoped that the present investigation will throw some light upon the fundamental principles involved and stimulate such further investigations as may be necessary for the development of a universally satisfactory procedure.

In the past the general tendency has been to lay the blame for discordant results upon the so-called "personal equation." In our experience the "personal equation," in so far as the actual manipulation of the test is concerned, does not enter into the problem at all. Any two workers, provided they are sufficiently experienced and dexterous with their hands to perform the necessary manipulations with accuracy and despatch, will obtain comparable results provided they are working with uniform materials throughout.

After watching the results of a large number of tests extending over a long period of time our attention was drawn to the fact that duplicate tests made with the same batch of culture

medium always gave uniform results, and that when discrepancies did occur, it was almost invariably when different batches of culture medium had been used for growing the test cultures or the subcultures. This led to the keeping of detailed records of all media used in this laboratory for the testing of disinfectants, including both the Rideal-Walker and Hygienic Laboratory broths. These records, which now cover a period of more than

TABLE 1

Variation in coefficient with different batches of media

Hygienic Laboratory method

DATE OF TEST	MEDIA FOR TEST CULTURES		SUB-CULTURE MEDIA, DATE MADE	COEFFICIENT	
	Date made	Amount of growth		Disinfectant A	Disinfectant C
1915	*1915*		*1915*		
July 2	June 28	Medium	July 1	15.00	5.25
July 3	June 28	Medium	July 1	15.25	5.50
July 5	June 28	Medium	July 2	15.00	5.47
July 6	June 28	Medium	July 2	15.25	5.47
July 7	June 28	Medium	July 2	15.06	5.50
July 8	July 2	Medium	July 6	15.12	5.37
July 10	July 2	Medium	July 10	15.50	5.25
July 12	July 3	Medium	July 10	15.00	5.50
July 13	July 10	Light	July 10	16.87	6.37
July 14	July 10	Light	July 10	16.62	6.41
July 15	July 10	Light	July 10	17.12	6.56
July 16	July 10	Very light	July 16	17.31	6.41
July 19	July 16	Medium	July 16	15.87	5.62
July 20	July 16	Medium	July 19	15.25	5.25
July 22	July 16	Medium	July 19	15.00	5.37
July 23	July 19	Light	July 20	17.12	6.67
July 24	July 19	Light	July 20	17.50	6.75
July 26	July 23	Medium	July 23	15.25	5.50

two years, include the following points: the serial number of each lot of media, the particular jar of meat extract and peptone used in its preparation, the batch of media used each day for growing the test cultures, the relative amount of growth upon test cultures, and the number of each lot of media used for subcultures. These records, which now include more than 1500 separate tests by both methods and more than 200 different

lots of culture media, have proven of the utmost value and have led to the firm conviction that a great majority of discordant results are due to variations in culture media. Never during this time has there been the slightest difficulty in checking our coefficients within very narrow limits when the same lot of culture medium was used. When on the other hand two or more lots of media were employed for check tests, wide variations have frequently occurred.

TABLE 2

Variation in coefficient with different batches of media
Rideal-Walker method

DATE OF TEST	MEDIA FOR TEST CULTURES		SUB-CULTURE MEDIA, DATE MADE	COEFFICIENT	
	Date made	Amount of growth		Disinfectant A	Disinfectant C
1915	*1915*		*1915*		
July 20	July 18	Heavy	July 19	18.00	6.50
July 21	July 19	Heavy	July 19	18.88	6.50
July 22	July 19	Heavy	July 19	18.00	6.25
July 24	July 19	Heavy	July 22	18.00	6.00
July 26	July 19	Heavy	July 22	18.00	6.00
July 27	July 22	Medium	July 22	20.00	7.50
July 29	July 22	Medium	July 22	21.10	7.00
July 30	July 22	Medium	July 29	19.90	7.50
August 2	July 22	Medium	July 29	20.00	7.25
August 3	July 29	Heavy	July 29	18.00	6.50
August 4	July 29	Heavy	August 3	18.00	6.50
August 6	July 29	Heavy	August 3	18.00	6.75
August 7	August 3	Medium	August 3	21.00	7.50
August 9	August 3	Medium	August 3	21.00	8.00
August 10	August 3	Medium	August 10	20.00	7.50
August 11	August 3	Medium	August 10	21.00	7.75
August 12	August 12	Heavy	August 12	18.88	6.50

Tables 1 and 2 show the record of a series of tests by both the Hygienic Laboratory and Rideal-Walker methods on each of two disinfectants. The tables speak for themselves and show clearly the variations which may be anticipated when different batches of media are used for check tests. Extreme care is used in the preparation, adjustment and sterilization of all media, and every precaution taken to have the procedure uniform in

every detail. Yet, in spite of the utmost care, different lots of media are at times sufficiently unlike to cause serious deviations in coefficients, this condition being evident with either the Hygienic Laboratory or Rideal-Walker method.

A careful study of the above tables will bring out the exceedingly important fact that, while slight differences in media used for growing the test organism previous to its employment in a test may cause serious discrepancies, the same differences in subculture media are of practically no significance. This has held true throughout all of our tests, so that it seems well demonstrated that, provided one has a uniform medium for growing the test culture, normal variations in the subculture medium are of little importance. It is true that differences in the subculture medium may cause a profound difference in the character of the chart obtained; that is, in the actual number of positive tubes shown in the subcultures from a given dilution. This influence, however, appears to be fairly uniform both for coal tar disinfectants and for the phenol control, so that there is no material change in the resulting coefficient.

In an effort to ascertain the cause of these variations in culture media the first consideration was, of course, the uniformity of the materials used in preparing the media. The salt used was in all cases the highest grade of C. P. product obtainable, so that it seemed safe to eliminate this factor from the problem. This leaves the water, beef extract, and Witte's peptone open to suspicion.

The Rideal-Walker method specifies the use of distilled water for the preparation of culture media, while the Hygienic Laboratory method specifies the use of tap water. Although it is claimed by some that tap water gives a medium more suited to the growth of bacteria, the writer, in view of the wide differences in the composition of various city water supplies as well as the seasonal and other variations occurring in a single supply, is convinced that its use is not desirable. A series of experiments performed by him extending over a period of several years indicated that media prepared with tap water were not in all cases comparable with those prepared with distilled water;

furthermore, the seasonal variations in the composition of the New York City water supply seemed to be sufficiently great to affect the uniformity of our phenol coefficient determinations. The only possible advantage in the use of tap water lies in its inorganic salt content. That a certain inorganic salt content is essential to any culture medium cannot be denied. This may, however, well be supplied by the use of sodium chloride in conjunction with the inorganic salts of the peptone and meat extract. Thus there seems to be no good reason for complicating the situation by employing tap water of unknown and variable composition. Throughout the work here reported distilled water was used in the preparation of all culture media. This factor could not, therefore, be concerned in any possible variation in the results obtained.

A careful study of the records described above has failed to show any indication that lack of uniformity in Witte's peptone was in any way responsible for the unreliable results often obtained with different lots of media. These results, which now include upwards of twenty-five different lots of peptone, together with the experiments to be described later, seem to show with reasonable certainty that for purposes of standardization Witte's peptone is a reliable and uniform product.

Liebig's Extract of Meat, on the other hand, does not appear to be nearly so satisfactory. Several jars have been found which invariably produced a culture medium giving very high coefficients. The proportions of salts and other ingredients of meat extract undoubtedly vary from time to time and are possibly responsible for some of the variations in culture media. In the writer's opinion, however, the greater part of the difficulty is caused by the presence in some jars of a considerable amount of fat. Chemical examinations have shown that those jars giving high coefficients contained over 50 per cent more ether extractive than the average for Liebig's extract of meat. The usual process of boiling culture media with caustic soda would convert this fat, partially at least, into soap. The powerful antiseptic action of soap, even in very high dilutions, is well known and is probably sufficient to explain the high results

obtained with these jars of extract. If, as will be recommended in this paper, the media be left with acidity unadjusted the presence of fat will have no effect; furthermore, it appears to be only an occasional lot of extract which proves unsatisfactory, so that if each new lot be checked against a disinfectant of known strength this source of error may be eliminated.

When we come to the actual manipulations used in the preparation of culture media, an extremely complex and involved problem is presented. This question is largely one of the influence of the kind and amount of acidity upon the growth of the test organism as well as upon the composition of the medium itself during the various stages of its preparation. Clark, in a recent article in the Journal of Infectious Diseases, July, 1915, clearly shows the fallacy of our present titrimetric methods. He points out the important influence of the hydrogen ion concentration upon the viability of bacteria, as well as upon the chemical composition of media, and shows conclusively that our present methods of adjusting the acidity of culture media by phenolphthalein give absolutely no information upon this all-important point.

The Rideal-Walker and Hygienic Laboratory broths form excellent examples of the differences in actual acidity (hydrogen ion concentration) which may be expected when media of different composition are brought to the same reaction with phenolphthalein by our present methods. The Rideal-Walker broth contains 20 grams of meat extract, 20 grams of peptone, and 10 grams of salt; while the Hygienic Laboratory broth contains 3 grams of meat extract, 10 grams of peptone and 5 grams of salt. The titration curves of these two solutions are shown below in Chart I.

The curves show the progressive changes in the hydrogen ion concentration of these solutions caused by successive additions of acid or alkali. Assuming the value $P_H = 8.5$ as the neutral point of phenolphthalein and calculating both from the point at which the curves cross this line we find that when the reaction has been brought to + 1.5 by the usual method, the Rideal-Walker broth would have a hydrogen ion concentration of ap-

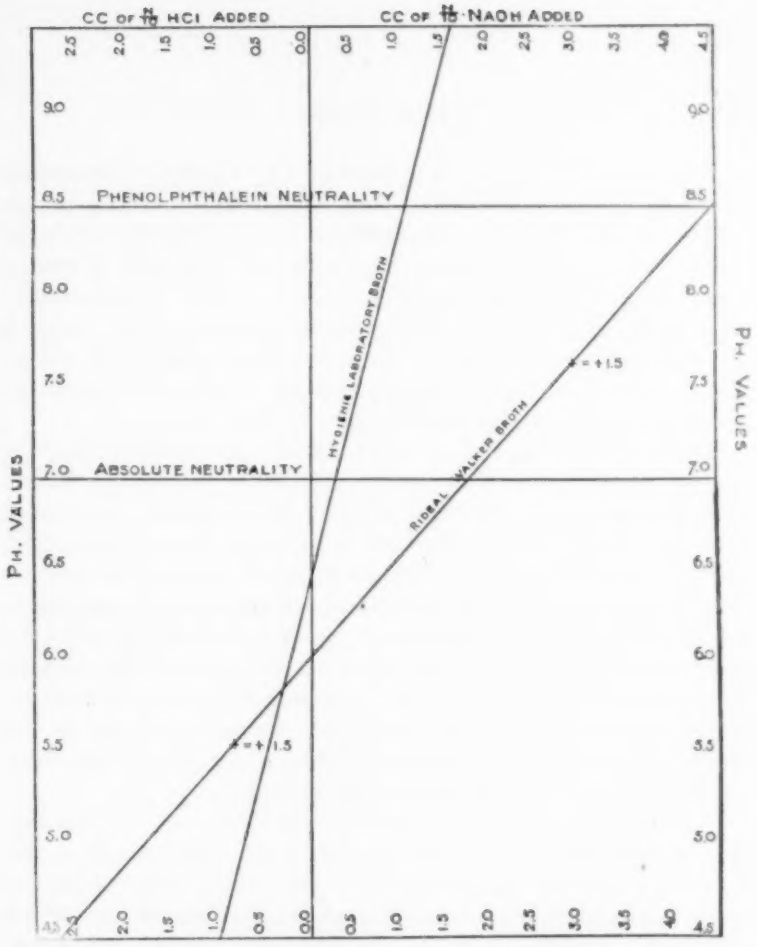


CHART 1. TITRATION CURVES OF 10 CC. OF HYGIENIC LABORATORY AND 10 CC. OF RIDEAL-WALKER BROTHS

The hydrogen ion concentrations given in this paper are expressed in the P_H values of Sörensen, (Compt. rend. du Lab. Carlsberg, 1909, **8**, p. 1.) The hydrogen electrode and a Leeds-Northrup potentiometer were used in the majority of the determinations. In some cases, however, the values given were obtained colorimetrically by means of the indicators, and essentially the same technique as described by Clark and Lubs (this Journal, vol. **2**, nos. 1, 2, and 3). We have made some rather extensive comparisons between this colorimetric procedure and the electrometric method as applied to the various media described in this paper and including the entire series of indicators recommended by Clark and Lubs. With all the sulphonaphthalein indicators the agreement has always been within 0.1 P_H. With methyl red, however, the results have been uniformly about 0.2 P_H more acid than those obtained with the hydrogen electrode. We believe, however, that for the majority of bacteriological purposes such an error is without significance and that the colorimetric method, carefully applied, constitutes a reliable and exceedingly convenient means for controlling the acidity of most types of culture media.

proximately $P_H = 7.65$ while that of the Hygienic Laboratory broth would be about 5.55. Due to the fact that few people judge the end point of phenolphthalein at exactly $P_H = 8.5$ the hydrogen ion concentration of media as ordinarily prepared varies somewhat from the above figures. This is particularly true of the Hygienic Laboratory broth in which the P_H value is usually very close to 5.0. Two media with such a wide difference in hydrogen ion concentration surely could not be expected to give comparable coefficients.

While the matter will be taken up in detail later, it may be well to mention here that the high results of the Rideal-Walker test as compared with the results of the Hygienic Laboratory test are not due to the difference in hydrogen ion concentration. The Rideal-Walker broth, with its 10 grams of NaCl in addition to the large amount introduced with 20 grams of meat extract, contains sufficient inorganic material to inhibit the growth of a weakened organism. If the salt be left out of the Rideal-Walker broth the results will be considerably lower than those obtained with the Hygienic Laboratory broth, in spite of the fact that the hydrogen ion concentration and titratable acidity of the Rideal-Walker broth are not changed.

While the above is sufficient to show the fallacy of attempting, by phenolphthalein titration, to adjust the reaction of culture media of different composition to the same point; there are many other points in our present methods of preparing culture media which make it practically impossible always to prepare even the same medium with a uniform hydrogen ion concentration. In a culture medium titrated with phenolphthalein there is no sharp end point. No two people will judge the "faint but distinct pink color" in exactly the same way. It is true that one person can, with practice, so train his eye as to obtain generally consistent results. There is no question, however, but that different workers, no matter how skillful and painstaking with their titration, cannot produce media of uniform reaction. This point is well illustrated in table 3.

After solution of the ingredients, a batch of Hygienic Laboratory medium was divided into three equal portions and

TABLE 3

Variations in coefficients obtained in different observers

ADJUSTED BY	FINAL TITRATION BY			HYGIENIC LABORATORY PHENOL COEFFICIENT OF DISINFECTANT A
	A	B	C	
A.....	1.48	1.91	1.26	$\frac{\frac{1100}{80}+\frac{1600}{100}}{2}$ = 14.75 average of 4 tests
B.....	1.21	1.56	1.08	$\frac{\frac{1100}{80}+\frac{1500}{100}}{2}$ = 14.37 average of 4 tests
C.....	1.75	2.17	1.41	$\frac{\frac{1300}{80}+\frac{1700}{100}}{2}$ = 16.62 average of 4 tests

each of three bacteriologists asked to adjust the acidity of one portion. After the media were completed each man was asked to determine the final reaction of the three lots without in any case knowing the identity of any of the samples. These results are shown above, together with the Hygienic Laboratory coefficients of coal tar disinfectant A resulting from the use of these media. The hydrogen ion concentration of the media was as follows.

Medium adjusted by bacteriologist A = 5.0
Medium adjusted by bacteriologist B = 5.6
Medium adjusted by bacteriologist C = 4.7

In the above determinations, as well as in all such tests reported in this paper, the medium used for growing the test culture and for subcultures was the same. The test organism was transferred daily for seven days and no longer, upon the particular medium indicated before being employed for a test. Great care was taken to have the test cultures in all cases exactly twenty-four hours old. We consider this an important point and are convinced that a few hours difference in the age of the test cultures has a very marked influence upon the uniformity of results. In order to eliminate every possible chance of variation, one jar of Liebig's extract of meat and one bottle

of Witte's peptone were set aside for use exclusively with the experimental media, so that all media described in this paper were made from the same jar of extract and the same bottle of peptone, unless otherwise indicated. In no case have conclusions been based upon the results of less than four tests, and usually six or more duplicate tests were made on different days and with different lots of media.

TABLE 4

Variation in coefficients with different methods of preparing media
Hygienic Laboratory technique

DISINFECTANT	DILUTION	MEDIUM: STANDARD R.-W. BROTH						MEDIUM: R.-W. BROTH, REDUCED TO + 1.5 WITHOUT NUETRALIZING					
		Minutes of exposure											
		2½	5	7½	10	12½	15	2½	5	7½	10	12½	15
A	1: 900							−	−	−	−	−	−
	1: 1000							+	−	+	−	−	−
	1: 1200							+	+	+	−	−	−
	1: 1300							+	+	+	+	+	+
	1: 1400	−	−	−	−	−	−	+	+	+	+	+	+
	1: 1600	+	−	−	−	−	−	+	+	+	+	+	+
	1: 1800	+	+	−	−	−	−						
	1: 2000	+	+	−	−	−	−						
	1: 2200	+	+	+	−	−	−						
	1: 2400	+	+	+	+	+	+						
Phenol	1: 80	−	−	−	−	−	−	−	−	−	−	−	−
	1: 90	+	−	−	−	−	−	+	+	−	−	−	−
	1: 100	+	+	−	−	−	−	+	+	+	+	−	−
	1: 110	+	+	−	+	−	−	+	+	+	+	+	+
	1: 120	+	+	+	+	−	−	+	+	+	+	+	+
	1: 130	+	+	+	+	+	+						
Coefficient		17.91						11.62					
P_H		7.8						8.4					

The Hygienic Laboratory method states simply that the medium should be adjusted to a reaction of + 1.5. A simple solution of the ingredients for the Hygienic Laboratory broth has an initial reaction of about + 0.9. Should this solution be neutralized and then brought up to + 1.5, or should sufficient acid be added to bring the reaction to the desired point without

neutralization? The Rideal-Walker broth has an initial acidity of + 3.5. This method specifies that the medium shall be neutralized and then 15 cc. of HCl per liter shall be added. It would seem therefore that there could be no excuse for simply adding sufficient alkali to bring the reaction down to the desired point.

TABLE 5

Variation in coefficients with different methods of preparing media
Hygienic Laboratory technique

DISINFECTANT	DILUTION	MEDIA: H.-L. BROTH, NEUTRALIZED AND THEN RAISED TO + 1.5 WITH HCl						MEDIA: H.-L. BROTH, RAISED TO + 1.5 WITH HCl WITHOUT NEUTRALIZATION					
		Minutes of exposure											
		2½	5	7½	10	12½	15	2½	5	7½	10	12½	15
A	1:1000	−	−	−	−	−	−	−	−	−	−	−	−
	1:1100	−	−	−	−	−	−	+	−	−	−	−	−
	1:1200	−	−	−	−	−	−	+	+	−	−	−	−
	1:1300	+	−	−	−	−	−	+	+	+	−	−	−
	1:1400	+	+	+	−	−	−	+	+	+	+	−	−
	1:1500	+	+	+	+	−	−	+	+	+	+	+	−
	1:1600	+	+	+	+	−	−	+	+	+	+	+	+
	1:1700	+	+	+	+	+	−	+	+	+	+	+	+
	1:1800	+	+	+	+	+	+	+	+	+	+	+	+
Phenol	1: 80	−	−	−	−	−	−	−	−	−	−	−	−
	1: 90	−	−	−	−	−	−	+	−	−	−	−	−
	1: 100	+	+	−	−	−	−	+	+	+	−	−	−
	1: 110	+	+	+	+	−	−	+	+	+	+	+	+
	1: 120	+	+	+	+	+	+	+	+	+	+	+	+
Phenol coefficient		14.94						13.75					
P_H value		5.2						5.7					

The effects of such differences in the preparation of culture media are illustrated by tables 4 and 5. In obtaining the results shown in these tables a single batch of Hygienic Laboratory broth and one of Rideal-Walker broth were prepared. Before adjusting the acidity, each lot was divided into two portions, one portion being neutralized with NaOH and then without filtration brought up to a reaction of + 1.5 with HCl, while to the other portion sufficient acid or alkali, as the case might be, was added to bring the reaction to the desired point. The differ-

ences in hydrogen ion concentration caused by such a variation in the method of preparing the media are also shown in the table. While this difference is not great, it is undoubtedly the cause of at least part of the discrepancies shown in the coefficients. It is, however, probable that a greater effect is produced by the changes in composition caused by the different methods of preparation.

When any medium is neutralized with alkali a considerable precipitate forms which only partially redissolves upon the addition of acid. This precipitate which separates out on the addition of acid or alkali consists of protein material and a considerable amount of phosphate, and its removal has a decidedly deleterious influence upon the subsequent growth of organisms. The hydrogen ion concentration of the medium is materially raised, due probably to the removal of "buffers." The increased hydrogen ion concentration, together with the reduced nutritive value, and the absence of phosphate, result in a very poor growth of the typhoid organism. On the finished culture being used for tests the results are not only much too high but the charts obtained are very irregular and unsatisfactory. This point is well illustrated by table 6. As would be expected, this phenomenon is more marked with the Hygienic Laboratory broth than with the Rideal-Walker medium.

It has probably been the experience of every worker that the amount of alkali indicated by titration is never sufficient to bring about complete neutralization of the media, it being necessary to add a considerable excess over the amount indicated. There will be a very decided difference in the coefficient obtained, depending upon how close to the neutral point the reaction has been carried before the addition of acid.

As previously mentioned, the Rideal-Walker method specifies that, after the medium has been neutralized, 15 cc. of N/1 HCl per liter shall be added. This should give the final product a reaction of + 1.5, but the acidity is always considerably less than the standard. Wide variations in hydrogen ion concentration with resulting variations in coefficients are obtained, depending upon whether or not an attempt is made to correct the medium to exactly 1.5.

In order to ascertain, if possible, just what influence variations in hydrogen ion concentration have upon culture media, a series of experiments was performed with media of varying P_H values. In these tests the proportion of the meat extract and peptone was as specified in the Hygienic Laboratory method, and in most cases the P_H values were adjusted by simply adding sufficient

TABLE 6

Variation in coefficients with different methods of preparing media
Hygienic Laboratory technique

DISINFECTANT	DILUTION	MEDIUM: H.-L. BROTH, NEUTRALIZED AND BROUGHT UP TO + 1.5						MEDIUM: H.-L. BROTH, NEUTRALIZED, FILTERED AND THEN BROUGHT UP TO + 1.5					
		Minutes of exposure											
		2½	5	7½	10	12½	15	2½	5	7½	10	12½	15
A	1:1200	−	−	−	−	−	−						
	1:1400	+	+	−	−	−	−						
	1:1600	+	+	+	+	−	−	−	−	−	−	−	−
	1:1800	+	+	+	+	+	+	+	−	−	−	+	−
	1:2000	+	+	+	+	+	+	+	+	−	−	−	−
	1:2200							+	+	−	−	+	−
	1:2400							−	+	+	+	−	−
	1:2600							+	+	+	+	+	+
Phenol	1: 80	−	−	−	−	−	−						
	1: 90	+	−	−	−	−	−						
	1: 100	+	+	+	−	−	−	−	−	−	−	−	−
	1: 110	+	+	+	+	+	−	+	−	−	−	−	−
	1: 120	+	+	+	+	+	+	+	+	−	+	−	−
	1: 130							+	+	−	−	+	−
	1: 140							+	+	+	+	+	+
Coefficient		14.77						17.72					
P_H		5.26						4.92					

NaOH or HCl, to bring the reaction to the desired point. Considerable work, however, was done in which the P_H value was adjusted by the use of phosphoric, lactic and citric acids, and sodium acid phosphate. Little difference could be detected in media prepared with different acids, provided the hydrogen ion concentration was uniform. In fact, all our work pointed to the conclusion that ordinary variations in the character and amount

of titratable acid present are of little importance, except in so far as they influence the P_H value.

The method of preparing the medium was as follows. The peptone, meat extract, and salt were boiled 15 minutes in the proper amount of distilled water. Then sufficient acid or alkali, as the case might be, was added to bring the P_H value to the desired point, the medium then being sterilized under 10 pounds pressure for twenty minutes. Different lots of medium were prepared in which the P_H values increased regularly by one-half unit from 8.5 to 4.5, the entire series being repeated three times. The relative amount of precipitate in each lot of medium was observed. A precipitate always formed upon the addition of more than a trace of alkali, and usually upon the addition of acid. In some instances the precipitate was not observable at once, but was present after the medium had been sterilized and allowed to cool. In all cases the amount of precipitate recorded is the amount present in the finished product.

It was found that the solubility of the peptone and meat extract was largely dependent upon the hydrogen ion concentration. This is graphically shown in curve 2, Chart II. When the P_H value fell between 6 and 7 no precipitate of any kind was present in the finished medium. A slight increase or decrease in acidity on either side of this point was however sufficient to cause a marked precipitation of the media. While the acidity of ordinary culture media probably never runs beyond $P_H = 4.5$, it is worthy of mention here that, when the P_H value is over 4, the peptone and meat extract again becomes perfectly soluble, the maximum precipitation occurring at approximately $P_H = 4.75$. It is also worthy of note that a chemical examination of these precipitates showed that that formed on the acid side of the chart consists almost entirely of protein, while that found on the alkaline side contains relatively large quantities of phosphates.[1]

[1] The writer feels that he should acknowledge his indebtedness to Dr. I. J. Kligler of the American Museum of Natural History, who, while discussing the results of some similar investigations of his own with an American peptone, first called the writer's attention to the fact that the solubility of peptone depended largely upon the hydrogen ion concentration of the solvent.

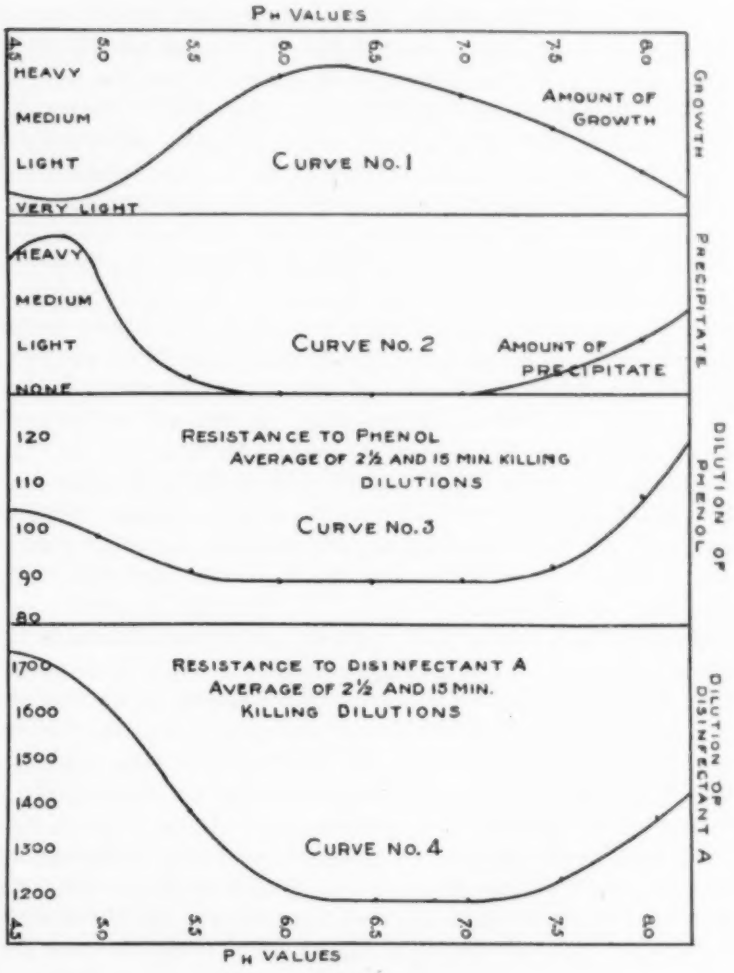


CHART 2

Examinations of a large number of different batches of media showed that the P_H value of standard Hygienic Laboratory broth varies from 4.75 to 5.75, while that of standard Rideal-Walker broth varies from 7.5 to 8.5. It will be seen therefore that the

composition of these media which is dependent largely upon a variable hydrogen ion concentration must consequently itself be variable. Such variations in chemical composition are undoubtedly one cause of the differences in the coefficients obtained with different lots of the same culture medium, although the indefinite hydrogen ion concentration is, of itself, without any reference to its influence upon solubility, an important point.

The development of the test organism in these media also forms an important index of their suitability for purposes of standardization. Five tubes of each broth were inoculated with *Bact. typhosum* Hopkins and were transferred daily for ten generations. These cultures were incubated at 37°C. and after twenty-four hours the amount of growth in each was recorded. The relationship between the amount of growth and the hydrogen ion concentration of the media is shown in curve 1, Chart II. The close agreement of this curve with that showing the relative amount of precipitate is quite remarkable and, as might be expected, the most luxuriant growth occurs in the same zone as that in which the peptone and meat extract are completely soluble.

Finally, these media were employed in determinations of the phenol coefficient of a coal tar disinfectant. At least five tests were run with each kind of medium on different days, using the same medium for both subcultures and for preliminary cultivation of the test organisms. The resistance of *Bact. typhosum* Hopkins in its relation to the various P_H values of the media is shown in curves 3 and 4, Chart II.

The curves plotted in each case were obtained by taking the mean of the two and one-half and 15 minute killing dilutions, as shown by the average result of not less than five check tests. It will be seen that the general outline of these curves corresponds with the two previous ones, the most important point being that between $P_H = 6$ and $P_H = 7$ the resistance of the organism is strong and constant, as indicated by the flatness of the curve. As the acidity is increased above $P_H = 6$ the resistance to phenol decreases slightly, while the resistance to the coal tar disinfectant decreases very rapidly, resulting in a

large increase in coefficient. On the other hand, when the acidity is reduced below $P_H = 7$ the resistance to phenol increases more rapidly than the resistance to a coal tar disinfectant, the result being a lowered coefficient. We have no explanation to offer for this phenomenon unless it be the fact that with increased acidity a large amount of protein material, but no phosphate, is removed from the media, while with a decreased acidity a large amount of essential phosphate and a comparatively small amount of protein is removed.

From these results it would seem, however, that we are at least justified in assuming that in order to obtain uniform results the hydrogen ion concentration of a culture medium should be between $P_H = 6$ and $P_H = 7$. This is further borne out by a study of the uniformity of coefficients obtained in duplicate tests with media of the same hydrogen ion concentration. Our results show that between $P_H = 6$ and $P_H = 7$ our phenol coefficients varied less than 10 per cent; between $P_H = 4.5$ and $P_H = 5.5$ our phenol coefficients varied from 30 to 40 per cent; while between $P_H = 7.5$ and $P_H = 8.5$ our coefficients varied from 20 to 25 per cent. In this connection it is of importance to note that a simple solution of the ingredients with no adjustment of the acidity whatever gives a P_H value most favorable for uniform results, namely, 6.3 to 6.7. It would seem, therefore, that there is no legitimate excuse for spoiling a good thing by juggling the reaction.

The Hygienic Laboratory broth has been often criticised on the ground that it contains insufficient nutriment for the normal growth of the typhoid organism, resulting in weakened and unreliable cultures. That this is not true is well demonstrated by our results, for when the hydrogen ion concentration of the culture medium is approximately $P_H = 6.5$ and the formula is as specified in the Hygienic Laboratory method *Bact. typhosum* grows luxuriantly and the cultures have been found to possess a remarkable degree of uniformity in their resistance to the action of disinfectants. The light, variable cultures of *Bact. typhosum* obtained with the regular Hygienic Laboratory broth are due not to a lack of a sufficient amount of nutriment, but to

an altered composition and high acidity brought about by a misguided attempt to adjust the reaction. However, it was thought wise to determine the effect of varying the proportion of different ingredients in culture media. A series of experiments was therefore performed as indicated in the following tables. In this work no attempt was made to adjust the acidity, the ingredients being simply dissolved, filtered, and sterilized; otherwise the details throughout the procedure were exactly the same as those previously employed.

TABLE 7

Showing effect upon culture media of varying amounts of Witte's peptone. All media in this table contained Liebig's Meat Extract, 3 grams, and NaCl, *5 grams, to each 1000 cc. of distilled water, in addition to the amount of peptone indicated*

AMOUNT OF WITTE'S PEPTONE PER LITER	PH VALUE	TITRATABLE ACIDITY TO PHENOLPHTHALEIN	RELATIVE AMOUNT OF GROWTH SHOWN IN PRELIMINARY CULTURES	RESISTANCE TO DISINFECTANT AVERAGE OF 2½ AND 15 MINUTE KILLING DILUTIONS		HYGIENIC LABORATORY COEFFICIENT OF DISINFECTANT A*
				Phenol	Disinfectant A	
grams.						
5	6.6	+0.75	Medium	1:95	1:1500	15.77
10	6.5	+0.93	Heavy	1:90	1:1200	13.33
15	6.4	+1.12	Heavy	1:89	1:1200	13.48
20	6.4	+1.35	Heavy	1:88	1:1190	13.52
25	6.3	+1.51	Heavy	1:89	1:1190	13.37
30	6.3	+1.74	Heavy	1:91	1:1210	13.29

* Average results of five check tests.

Table 7 shows the influence of adding varying amounts of Witte's peptone to culture media, the proportions of the other ingredients remaining constant. One medium was prepared containing no peptone. In this medium *Bact. typhosum* grew very poorly indeed, so that it was not considered worth while to continue the experiment. With 5 grams of the peptone a good growth of the test organism was obtained. The resistance of the organism was, however, markedly weaker than when grown in any other medium in this series. The use of amounts of Witte's peptone varying from 10 to 20 grams per liter made no apparent difference in the growth of the organism nor in

its relative resistance to the action of disinfectants. It would seem, therefore, that there is no good reason for using a larger quantity of this ingredient than is now called for by the Hygienic Laboratory method.

That some meat extract is essential is shown by the results obtained with the first medium in table 8. Where no extract was used it was impossible to obtain more than an exceedingly light growth of the test organism, and when such a culture was used for testing a disinfectant the coefficient was not only

TABLE 8

Showing effect upon culture media of varying amounts of Liebig's extract of meat. All media in this table contained Witte's peptone, 10 grams and NaCl, *5 grams, to each 1000 cc. of distilled water in addition to the amount of meat extract indicated*

AMOUNT OF LIEBIG'S EXTRACT OF MEAT PER LITER	P_H VALUE	TITRATABLE ACIDITY TO PHENOLPHTHALEIN	RELATIVE AMOUNT OF GROWTH SHOWN IN PRELIMINARY CULTURES	RESISTANCE TO DISINFECTANT AVERAGE OF 2½ AND 15 MINUTE KILLING DILUTIONS		HYGIENIC LABORATORY COEFFICIENT OF DISINFECTANT A*
				Phenol	Disinfectant A	
grams						
None	6.9	+0.29	Very light	1:99	1:1710	17.27†
3	6.5	+0.81	Heavy	1:89	1:1190	13.37
5	6.4	+1.14	Heavy	1:91	1:1280	14.06
10	6.2	+1.81	Heavy	1:93	1:1380	14.85
15	6.2	+2.62	Heavy	1:93	1:1490	16.02
20	6.1	+3.44	Medium	1:95	1:1510	16.52
25	6.1	+4.23	Medium	1:97	1:1650	17.01

* Average results of five check tests.

† Very irregular.

very high but the charts obtained were very irregular and unsatisfactory in every particular. With 3 grams of meat extract the growth of the test organism was fully as good as that obtained with any medium tested. Its resistance to the action of disinfectants was strong and uniform, so that there was no difficulty in getting satisfactory checks with duplicate tests. As the amount of meat extract was increased there was, apparently, a steady decrease in the resistance of the test culture, and as this was more pronounced in the case of the coal tar disinfect-

ants than with phenol there was a corresponding increase in the phenol coefficient. This weakened resistance, however, is more apparent than real and is due largely to the fact that media containing large percentages of meat extract are sufficiently antiseptic to inhibit the growth of an organism which has been weakened but not killed by exposure to disinfectants. This is shown by a series of experiments in which the second medium in this table was used for subcultures with test cultures grown in each of the media shown in the table. In these experiments there was also an increase in coefficient with an increased percentage of meat extract. This, however, was only about one-

TABLE 9

Showing effect upon culture media of varying amounts of NaCl. *All media in this table contained Witte's peptone, 10 grams and Liebig's Extract of Meat, 3 grams, to each 1000 cc. of distilled water in addition to the amount of* NaCl *indicated*

AMOUNT OF NaCl PER LITER	P_H VALUE	TITRATABLE ACIDITY TO PHENOL-PHTHALEIN	RELATIVE AMOUNT OF GROWTH SHOWN IN PRELIMINARY CULTURES	RESISTANCE TO DISINFECTANT AVERAGE OF 2½ AND 15 MINUTE KILLING DILUTIONS		HYGIENIC LABORATORY COEFFICIENT OF DISINFECTANT A*
				Phenol	Disinfectant A	
grams						
None	6.6	0.93	Medium	1:94	1:1410	15.00
5	6.5	0.93	Heavy	1:90	1:1191	13.22
10	6.5	0.94	Medium	1:94	1:1480	15.74
15	6.4	0.93	Medium	1:96	1:1590	16.56
20	6.3	0.94	Light	1:98	1:1670	17.02

* Average result of five check tests.

half that obtained when the higher amounts of meat extract were introduced in the subculture media as well as in that used for preliminary cultivation. Moreover, this inhibitive action seems to vary materially with different lots of Liebig's extract of meat, so that it would seem wise to reduce this variable factor to a minimum by employing for a standard method as small an amount of meat extract as is consistent with the needs of the organism. From our results it seems clear that 3 grams per liter is ample.

It will be noted that in table 8 there is a very marked increase in titratable acidity when the larger amounts of meat extract

are used and at the same time there is a slight increase of the hydrogen ion concentration. It may possibly be contended that the inhibitive influence of the meat extract is due to this increased acidity. Such, however, is not the case as was shown by the fact that when the titratable acidity of these media was in all cases reduced to + 1.0 the resulting coefficients, while somewhat lower as would be expected with the decreased P_H value, still showed the same steady increase with increased percentages of meat extract.

That some salt is desirable in culture media is shown by the results in table 9, the best growths and the most resistant cultures being obtained with the medium containing 0.5 per cent. As the percentage of salt was increased the growth became less luxuriant with a corresponding increase in the phenol coefficient. As in the case of meat extract, the increased coefficient is partly due to the inhibitive action of the larger amount of salt upon the exposed organism rather than to an actual lowering of its resistance. In this connection it is of interest to note that, as Liebig's Meat Extract contains over 30 per cent of inorganic salts and Witte's peptone 3 to 10 per cent, a medium made up of 3 grams of the former, 10 grams of the latter, and 5 grams of NaCl would have an inorganic salt concentration closely approximating that of physiological salt solution. This is significant in view of the fact that this medium, of all those tested, has been the most favorable for the growth of the *Bact. typhosum* and has in every respect given the most uniform and satisfactory results.

A medium such as the above, in which no attempt is made to adjust the acidity, always has, in our experience, a P_H value of 6.3 to 6.6 which has been shown to be within the zone of hydrogen ion concentration most conducive to uniform and reliable results. The percentages of peptone, meat extract, and salt are those which have been found most favorable for a normal unrestricted growth of the test organism, as well as least liable to introduce disturbing variations. The phenol coefficient obtained with this medium is considerably lower than that given by either of the old methods, and hence may be considered un-

desirable by some manufacturers of disinfectants. However, if all manufacturers would guarantee their products upon the same basis, it is hard to see where anyone would suffer an injustice.

The most important need in a standard method for control of disinfectants is, of course, a reliable procedure for the accurate determination of the relative germicidal strength of disinfectants of the same class, and it makes no particular difference whether the results be high or low, so long as the results are uniform and the actual relative differences between different products are clearly shown. This seems to be easily accomplished if a culture medium such as the above be employed. Up to the present time more than 150 different determinations of phenol coefficients upon several disinfectants have been made with this medium, including the use of 50 different batches of media and 12 lots each of Witte's peptone and Liebig's extract of meat. The results of these tests have been very satisfactory throughout; the experimental error shown by duplicate tests has never exceeded 8 per cent and has almost always been much less than 5 per cent. It is hoped that, provided these results are confirmed, such a culture medium may be adopted as an official standard.

The writer is of the opinion that it is of little importance whether one employs the Hygienic Laboratory or the Rideal-Walker technique, provided a proper culture medium be used; personally, we are slightly inclined to favor the Hygienic Laboratory procedure and feel that the results obtained with it are slightly more uniform than with the Rideal-Walker. It is also quite possible that some entirely new technique may be developed which will prove superior to either of the present procedures. Whether old or new technique finally prevail, a reliable culture medium is the first essential.

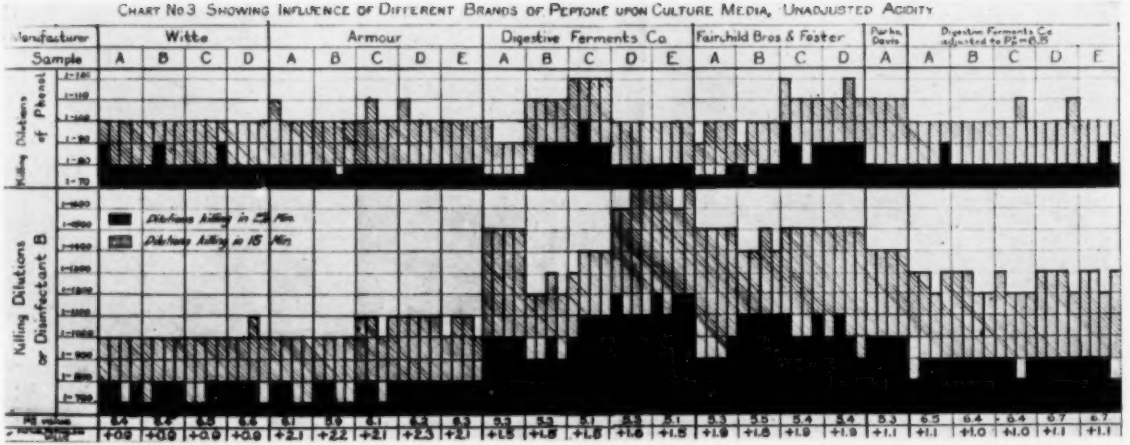
Witte's peptone, owing to present European conditions, is very hard to obtain, and the small quantities available are almost prohibitive in price. As it is uncertain when a new supply will be available upon the American market, it would be of the utmost value if a product of American manufacture could

be found which might safely be substituted for Witte's. In order that data on this subject might be available for the use of the Committee on Standardization of Disinfectants of the American Public Health Association a study of various American peptones was undertaken, in conjunction with Prof. Earle B. Phelps, Chairman of the above committee.

The different manufacturers were asked to submit samples representing as many different batches of their product as possible. In all, fifteen samples from four manufacturers were received, all of which were examined in comparison with Witte's peptone. For this work media were prepared from the different peptones, using the formula previously described as having given the most uniform results with Witte's; namely, 10 grams of peptone, 3 grams of Liebig's extract, and 5 grams of salt to each liter of distilled water, no attempt being made to adjust the acidity. The details of the experiments were in each case the same as those employed in the previous work. The influence of the different peptones upon the resistance of *Bact. typhosum* to phenol and to a coal tar disinfectant is shown graphically in Chart III. The solid portions represent those dilutions which kill in two and one-half minutes, while the shaded portions represent those dilutions which kill at fifteen minutes. The results of four separate tests with each lot of peptone are shown. The hydrogen ion concentrations, as well as the titratable acidities to phenolphthalein, are indicated on the chart. For comparative purposes similar results obtained with different lots of Witte's peptone are also included.

It will be noted that the hydrogen ion concentrations of the medium prepared with Armour's peptone varied from $P_H = 5.9$ to $P_H = 6.3$, thus practically falling within the zone where our previous work would lead one to expect uniform and reliable results. With these media the preliminary growth of the test organism was luxuriant. The resistance of the test organism, both to the action of phenol and to disinfectant B is remarkably uniform and in close agreement with the results obtained with Witte's peptone. In so far as can be determined from the limited number of samples examined, different lots of Armour's

CHART No 3 SHOWING INFLUENCE OF DIFFERENT BRANDS OF PEPTONE UPON CULTURE MEDIA, UNADJUSTED ACIDITY


Manufacturer
Witte
Armour
Digestive Ferments Co
Fairchild Bros & Foster
Parke Davis
Digestive Ferments Co adjusted to PH = 6.8
Sample
A B C D A B C D E A B C D E A B C D A A B C D E
Killing Dilutions of Phenol
1=110
1=100
1=90
1=80
1=70
Killing Dilutions or Disinfectant B
1=1600
1=1500
1=1400
1=1300
1=1200
1=1100
1=1000
1=900
1=800
1=700
Dilutions killing in 2½ Min.
Dilutions killing in 15 Min.
PH value
5.4 5.4 5.6 5.6 6.1 6.0 6.1 6.2 6.3 5.3 5.2 5.1 5.3 5.1 5.3 5.0 5.4 5.6 5.3 6.5 6.4 6.4 6.7 6.7
+0.9 +0.9 +0.9 +0.9 +2.1 +2.2 +2.1 +2.3 +2.1 +1.5 +1.5 +1.6 +1.6 +1.5 +1.9 +1.8 +1.9 +1.9 +1.1 +1.1 +1.0 +1.0 +1.1 +1.1

peptone seemed to possess a sufficient degree of uniformity, so that in this medium the product may possibly prove a satisfactory substitute for Witte's peptone. It must be remembered, however, that only five samples were examined, and it is quite possible that other lots may not prove so satisfactory.

Media prepared from the 5 samples of peptone furnished by the Digestive Ferments Company had a hydrogen ion concentration of from 5.1 to 5.3; media prepared from the 4 peptones furnished by Fairchild Brothers & Foster had a hydrogen ion concentration of from 5.3 to 5.5; while that from the one sample furnished by Parke, Davis & Company[2] had a hydrogen ion concentration of 5.3. It will be seen, therefore, that the acidities of these media lie outside of the zone in which uniform results were obtained with Witte's peptone, and as might be expected from the high acidity the results obtained with these products were much less satisfactory than those obtained with Armour's. The resistance of the test culture to the action of phenol was irregular and as a rule quite low. The resistance to the action of disinfectant B was also irregular and at the same time was very much less than when either Armour's or Witte's peptones were used. It is of interest to note that while all of these products were practically completely soluble in cold water, there was almost invariably a heavy precipitate in the finished sterilized medium, and that the growth of *Bact. typhosum* obtained was in all cases much less luxuriant than that given by either Armour's or Witte's peptones. This checks up very well with our previous results, which indicated that a medium having a hydrogen ion concentration in the neighborhood of 5 was variable in composition and in every way unsatisfactory for a normal development of the test organisms.

It was thought desirable to determine the effect of reducing the hydrogen ion concentration of these media to a point approximating that given by Witte's peptone. For this purpose the titration curves of batches of media prepared from each of the

[2] Only one sample of peptone was furnished by this company and it is understood that the preparation of this product is in an experimental stage and that it is not being marketed.

Digestive Ferments Company's peptones were determined. From these curves the amount of alkali necessary to bring the P_H value to 6.5 was calculated. Various lots of media were then prepared in which the ingredients were mixed and the amount of alkali indicated by the titration curve added, after which the mixture was boiled for fifteen minutes, filtered, tubed, and sterilized. As in the previous experiments, these media were then employed in the determination of the phenol coefficient of disinfectant B. These results are also shown in Chart III together with the final hydrogen ion concentrations, which will be seen to approximate closely the desired point.

The test organism grew luxuriantly, the growth obtained being fully equal to that given with the unadjusted medium prepared from Witte's or Armour's peptone. There was no evidence of any precipitate in the finished media, and as will be seen from the chart the resistance of the test organism was very uniform in all cases, so that the actual variations in coefficient are negligible. It will be noted, however, that the resistance to disinfectant B is still somewhat weaker than was shown with either Witte's or Armour's peptone and as a result the phenol coefficient is somewhat higher. Thus while the adjustment to a P_H value of approximately 6.5 of media prepared from the Digestive Ferments Company's peptone enables one to obtain uniform coefficients, these coefficients are not strictly comparable with those obtained with Witte's and Armour's. It seems quite clear, therefore, that there are differences in peptone other than acidity which are capable of causing variations in the resistance of *Bact. typhosum* to the action of disinfectants. While we have not taken the time to repeat these experiments on Fairchild Brothers & Foster's, or Parke, Davis & Company's peptones, we see no reason why a similar test should not give similar results.

From these results it becomes apparent that with the possible exception of one peptone, the American products cannot be safely substituted for Witte's peptone under the above conditions. It should be borne in mind, however, that these results were obtained upon culture media in which no attempt was

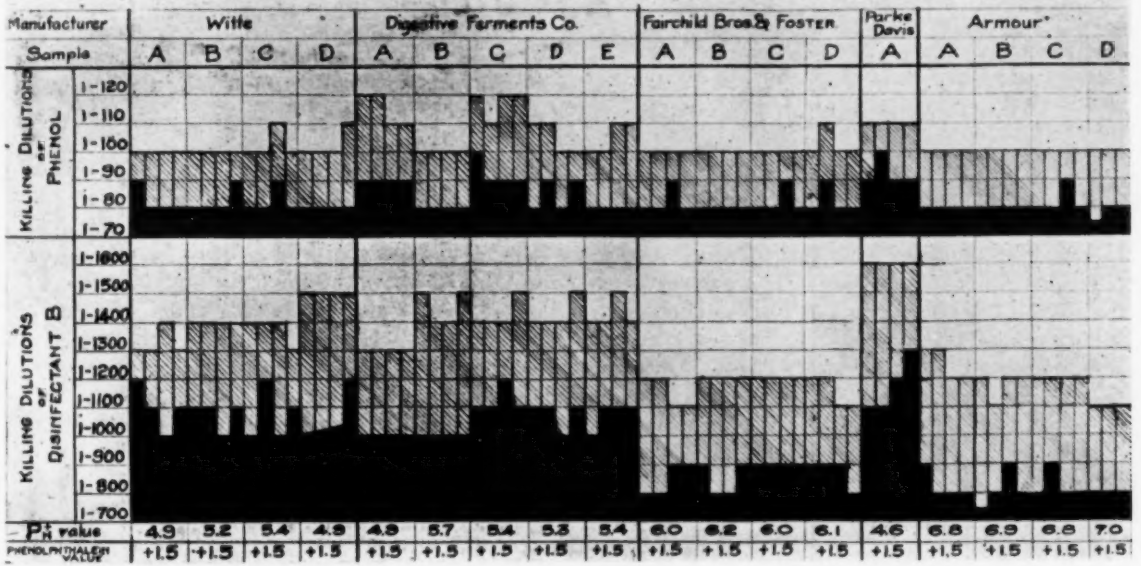
made to adjust the acidity. When the acidity is adjusted by first neutralizing and then bringing up to an acidity of + 1.5 to phenolphthalein entirely different results are to be expected. The results obtained with media adjusted in this way are shown in Chart IV.

A comparison of this chart with Chart III will bring out a remarkable difference in the behavior of the various peptones under these conditions. No better illustration could be desired of the futility of attempting to adjust the acidity of culture media to a definite point by phenolphthalein titration. Here we have sixteen different lots of culture media, all with a phenolphthalein acidity of + 1.5, in which the hydrogen ion concentration varies from $P_H = 4.6$ to $P_H = 7.0$. In the case of Witte's and Parke, Davis & Company's peptones, with which the initial titratable acidities were less than + 1.5, there is a marked increase in the hydrogen ion concentration. The media from Fairchild Brothers & Foster's and Armour's peptones, having an unadjusted acidity to phenolphthalein greater than + 1.5, show when adjusted to this point reduced P_H values. The Digestive Ferments Company's peptone, with which the initial phenolphthalein acidity was practically + 1.5, is not particularly affected except that there is a slightly greater variation in the P_H values of the adjusted media, which again illustrates the fallacy of our present method of adjusting the reactions of culture media.

Chart IV shows the same general relationship between the resistance of the organism to the action of disinfectants and the P_H value of the medium as was found with the unadjusted media given in Chart III. With the exception of greater variability in the phenol results, the resistance of the test organism when grown in media prepared with Digestive Ferments Company's peptone is practically identical with that shown by Witte's peptone. Fairchild Brothers & Foster's and Armour's peptones, owing to their low hydrogen ion concentration, cause a marked increase in the resistance of the test culture.

It will be noted that with adjusted culture media made from the Digestive Ferments Company's peptone the cultures are

CHART No4, SHOWING INFLUENCE OF DIFFERENT BRANDS OF PEPTONE UPON STANDARD HYGIENIC LABORATORY CULTURE MEDIA.



slightly more resistant and at the same time more uniform than with the unadjusted media. This certainly cannot be explained on the basis of hydrogen ion concentrations and the difference is too great to be laid to accident. This phase of the problem is worthy of careful study and involves painstaking investigations

TABLE 10

Showing phenol coefficients of disinfectant B obtained with different peptones

PEPTONE		AMOUNT OF GROWTH SHOWN IN PRELIMINARY CULTIVATION		PHENOL COEFFICIENT OF DISINFECTANT B AVERAGE RESULT OF FOUR TESTS	
		Medium adjusted	Medium unadjusted	Medium adjusted	Medium unadjusted
Witte	A	Light	Heavy	13.28	9.72
	B	Light	Heavy	13.45	9.72
	C	Light	Heavy	12.97	9.58
	D	Light	Heavy	13.28	10.00
Digestive Ferments Company	A	Medium	Light	11.21	14.92
	B	Light	Light	13.61	10.88
	C	Light	Light	12.75	11.52
	D	Light	Light	13.44	15.41
	E	Light	Light	13.78	15.83
Fairchild Brothers & Foster	A	Heavy	Medium	10.95	13.79
	B	Heavy	Medium	11.38	14.06
	C	Heavy	Medium	11.56	12.59
	D	Heavy	Medium	10.94	12.34
Armour	A	Heavy	Heavy	11.52	9.58
	B	Heavy	Heavy	11.04	9.79
	C	Heavy	Heavy	11.09	10.13
	D	Heavy	Heavy	10.58	10.41
	E	Heavy	Heavy		10.22
Parke, Davis & Company	A	Medium	Medium	13.21	12.70

into the chemistry of peptones. It does not, however, come within the scope of the present paper.

The average phenol coefficient obtained with each of the above peptones is shown above in table 10.

From a study of these results it seems clear that, with the possible exception of Armour's, none of the American peptones

could be safely substituted for Witte's in unadjusted culture media. The results obtained with Armour's peptone are fairly uniform and are in sufficiently close agreement with Witte's to warrant a more extended investigation of this product and to justify the hope that it may prove a satisfactory substitute for the imported peptone for use in unadjusted media, which we trust may soon be adopted as a standard for this work.

It will undoubtedly be some time, however, before such a change can be made in the official procedure. It would be of value, therefore, if an American product could be found which would give results comparable to and at least no worse than those given by Witte's peptone in the present Hygienic Laboratory media. A study of our results will show that the Digestive Ferments Company's peptone is the only one which could possibly fulfill these requirements and even with this the results are much less uniform than with Witte's. In justice to this product it should be stated, however, that the results given for Witte's peptone are from lots of media selected from a long series of tests and represent the average results which may be expected with this product, rather than the actual limits of variability. It should also be pointed out that these variations do not represent actual differences between the different samples of the same product. We have found in different batches of media made from the same bottle of Digestive Ferments Company's peptone variations fully as great as are shown here with different samples. The trouble does not appear to be due so much to actual variations in the peptone itself, as to the fact that with a high hydrogen ion concentration it is practically impossible to control the procedure so as always to produce solutions of uniform characteristics, even from identical materials.

SUMMARY

The essential points brought out by this work may be summed up as follows.

1. Variations in culture media are the cause of the majority of the discrepancies obtained in the bacteriological examination of disinfectants.

2. There is no indication that these variations in culture media are in any way due to lack of uniformity in Witte's peptone. Liebig's Extract of Meat, however, should be regarded with suspicion.

3. The greater part of the difficulty lies in our present methods of adjusting the acidity.

4. The hydrogen ion concentration of a culture medium has important influences upon its composition and upon its suitability for the growth of *Bact. typhosum*.

5. There is a marked relationship between the hydrogen ion concentration of the culture medium and the resistance of the test organism to the action of disinfectants.

6. The most satisfactory and uniform results have been obtained with a culture medium in which the P_H value falls between 6 and 7. This condition is easily obtained with a medium containing 10 grams of Witte's peptone, 3 grams of Liebig's meat extract, and 5 grams of salt, boiled fifteen minutes, filtered, tubed, and sterilized, with no attempt to adjust the acidity.

7. It has been found that 3 grams of meat extract are ample and that an increased amount is liable to cause disturbing variations.

8. It has also been found that 10 grams of Witte's peptone is sufficient for all the needs of the test culture.

9. The best results have been obtained with a medium containing 5 grams of salt per liter, an increase of salt content causing an increased coefficient due to the inhibitive action of the salt upon the exposed organism.

10. Of the different brands of American peptone only one has been found which could possibly be safely substituted for Witte's in the unadjusted medium. Insufficient samples, however, have been tested to enable one to make this statement with certainty.

11. The Digestive Ferments Company's peptone gives results most closely approximating those of Witte's when used in standard Hygienic Laboratory media.

In conclusion it should be stated that this work was made

possible by the financial support and interest of the West Disinfecting Company through their chemist, Dr. W. Dreyfus. The writer's appreciation is also due to Dr. H. D. Pease of the Lederle Laboratories for his interest in the work, and to Prof. E. B. Phelps of the Hygienic Laboratory, Washington, D. C., who very kindly had many determinations of hydrogen ion concentration made in his laboratory.

Since this article was written the Digestive Ferments Company and Fairchild Brothers & Foster have submitted new samples of peptone based upon the results of our experimental work. In these peptones they have attempted to standardize the hydrogen ion concentration so that the finished media with unadjusted acidity will have a desirable P_H value. We are at present examining these products, and while we have not as yet done a sufficient amount of work to warrant drawing definite conclusions, the present indications are that the new products will prove much more satisfactory than the old ones.

STUDIES ON THE NOMENCLATURE AND CLASSIFICATION OF THE BACTERIA

III. THE FAMILIES OF THE EUBACTERIALES

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From the Bacteriological Laboratories, Iowa State College, Ames, Iowa

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The order Eubacteriales may be characterized as follows:

Order 1. **Eubacteriales,** ordo nov.

Synonyms:

Bacterina Perty (1852, p. 179, in part)
Eubacteria Schroeter (1882, p. 154)
Eubacteria Migula (1900, p. 1)
Eubacteriaceae A. J. Smith (1902, p. 270)
Haplobacteriacei Fischer (1895), in part
Haplobacterinae Fischer (1903), in part

This order includes the true bacteria, those forms which are least differentiated and least specialized. The cells are usually minute, spherical, rodshaped or spiral, not typically producing true filaments although the cells may occur in chains or other groups. The cells do not have a well-organized or differentiated nucleus. They may be motile by means of flagella, or non-motile, never notably flexuous. Multiplication by transverse fission, never by longitudinal. Some forms, particularly rod-shaped types, produce endospores, but never conidia. Branching of cells occurs in a few forms which intergrade with the Actinomycetales. Neither sulphur granules nor bacteriopurpurin are present, though the cells may be pigmented. Chlorophyll is absent (with the possible exception of one genus.) The cells may be united into gelatinous masses, but never form motile pseudoplasmodia, nor develop a highly specialized cyst-producing fruiting stage.

With very few exceptions, authors have recognized that the group here termed the order *Eubacteriales* should be divided into three subgroups or families on the basis of the shape of the cell, that is, into cocci, bacilli and spirilla. Cohn (1872) used the names *Sphaerobacteria*, *Microbacteria*, *Desmobacteria* and *Spirobacteria* to designate the cocci, the short rods, the long rods and the spirilla respectively. Zopf (1883) used the names *Coccaceen* and *Bacteriaceen*, later (1885) including the spiral forms with the rods under the second family. Flügge (1886) termed the three groups *Mikrobacterien*, *Bacillen* and *Spirillen*. Schroeter (1886) used *Coccacei* and *Bacteriacei* in the sense of Zopf. Trevisan (1885) and De Toni and Trevisan (1889) include all spherical organisms under *Coccogenae* and all rods and spirals under *Baculogenae*. Sternberg (1892) employed the terms *Micrococci*, *Bacilli* and *Spirilla*. Migula (1894, 1895, 1897, 1900, 1904) uses the family names *Coccaceae*, *Bacteriaceae* and *Spirillaceae*. In this nomenclature he is followed by Lehmann and Neumann (1896), Chester (1897, 1901), A. J. Smith (1902), Kendall (1902), E. F. Smith (1905), Schneider (1912), Engler (1912), and Vuillemin (1913).

Hueppe (1895) termed the families *Coccaceae*, *Bacteriaceae* and *Spirobacteriaceae*. Matzuschita (1902), Fischer (1903) and Flügge (1905), use *Coccaceae*, *Bacillaceae* and *Spirillaceae*.

The principal departure from this type of classification is that of Jensen. As previously noted, this author does not recognize a group corresponding to the *Eubacteriales*. The families which he lists that would fall under this group as here defined would be *Oxidobacteriaceae*, *Luminobacteriaceae*, *Reducibacteriaceae*, *Acidobacteriaceae*, *Alkalibacteriaceae*, *Butyribacteriaceae* and *Putribacteriaceae*.

It is evident from the names which have been used that most authors have adhered very strictly to morphology as a basis for separation of families, while Jensen has emphasized physiology. It would seem that wherever a morphological basis for grouping does not lead to the separation of closely related forms, and the inclusion of unlike types in the same group, it should be employed, but when such occurs another method of separation,

probably based upon physiological characters should be used. The unanimity with which the separation into families on the basis of shape has been used would indicate that in general it has proved fairly satisfactory. In a few cases it probably leads to somewhat anomalous results. For example, to any student of the lactic acid bacteria the separation of the lactic streptococci and bacilli into different families is difficult in view of the numerous intergradations. Perhaps even more striking is the inclusion in separate families of Winogradsky's *Nitrosococcus* and *Nitrosomonas* in most classifications. The organisms belonging to the two genera are evidently closely related, they are both prototrophic, oxidize ammonia to nitrates, and do not develop on ordinary media. It would seem that these genera together with *Nitrobacter* might well be grouped together as a distinct family.

Many of the names which have been proposed for these families do not conform to the rule of the botanical code which reads "Families are designated by the name of one of their genera or ancient generic names with the ending *aceae*." With this rule in mind and with due respect to priority, the family names may be designated as *Coccaceae*, *Bacteriaceae*, *Spirillaceae* and *Nitrobacteriaceae*.

Following is a key to these families, giving the principal characteristics.

Key to the families of the Eubacteriales

A. Organisms usually growing more or less readily upon organic media, not securing growth energy primarily by the oxidation of ammonia or nitrites.
  I. Cells typically spherical.....Family I. *Coccaceae*
  II. Cells not spherical, elongate.
    a. Cells not spiral.....Family II. *Bacteriaceae*
    b. Cells spiral, or at least curved.....Family III. *Spirillaceae*

B. Not growing readily or at all on media containing considerable amounts of organic material; nitrifying bacteria, securing growth energy primarily by the oxidation of ammonia or nitrites.
  Cells may be either spherical or rod-shaped,
  Family IV. *Nitrobacteriaceae*

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THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE PRODUCTION OF PRECIPITATES IN A SOLUTION OF PEPTONE AND ITS RELATION TO THE NUTRITIVE VALUE OF MEDIA

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It is a well-known fact that when media are neutralized to phenolphthalein a precipitate is produced which may be partly redissolved by the addition of acid. If this precipitate is filtered off the nutritive quality of the medium is appreciably lowered. Likewise, experience has taught us that the optimum reaction of a culture medium is +1.0 to phenolphthalein or neutral to litmus. In neither case, however, has an adequate explanation been given for the observed facts.

From previous experiments on synthetic media it became evident that the significant nutritive factor in bacteriological culture media was the peptone and that a careful study of this substance should yield explanations of many puzzling facts. From various analyses it appears that petpone consists of proteoses, amino-acids and salts, the most important of the latter being the phosphates. The proteoses and complex nitrogenous compounds can not be utilized by the common bacteria but they do play an important part as buffers in the medium. The amino-acids are the essential nitrogenous foods upon which the nutritive value of the peptone depends. The phosphates furnish an important salt but are of even greater value as reaction regulators. An ideal peptone would, therefore, be one that contains these ingredients in the right proportions.

Since the reaction of the medium, both in the making and in the finished product, has so great an influence on the nutritive quality, attention was directed to the effect of different reactions,

in terms of hydrogen ion concentration, upon the peptone. Solutions of 1 per cent peptone in distilled water were made up and varying amounts of acid or alkali added to obtain a graded series of hydrogen ion concentrations. Solutions of Witte and Difco peptone were studied in this manner.

The results obtained bear so directly on the problem that even though the procedure was only qualitative they are of sufficient interest to deserve recording. Briefly stated, it was found that peptone has two precipitating zones, one on the acid side, the other on the alkali side, with an intermediate zone of complete solubility. In terms of P_H, peptone (Witte's) is completely soluble between P_H 6.8 and P_H 8.0. At P_H 8.2 precipitation begins and increases progressively to P_H 9.0. Similarly, a precipitation occurs at about 5.4 and increases up to about 5.0, then decreases again to about P_H 4.0 when no precipitate is formed.

The nature of the precipitate in the two zones is suggestive. At the alkali end the precipitate appears to consist largely of phosphates plus some organic constituents. The acid precipitate, on the other hand, is organic and when redissolved gives reactions characteristic of proteoses and peptone.

Both Witte's and Difco peptone behaved in the manner outlined with the following exceptions: Witte's peptone when dissolved in water has a P_H value of 6.5 to 6.8. It dissolves slowly and with a clear solution. On autoclaving only a slight sediment is sometimes obtained. The Difco peptone, on the other hand, has a P_H value of 5.1 to 5.4 when dissolved. It goes into solution readily but gives a heavy precipitate on autoclaving. This is to be expected since its reaction is right at the precipitating zone. Like Witte's peptone it has a clear solubility zone between P_H 7.0 and 8.0 and a precipitating zone beyond 8.0.

The bearing of these facts on the changes obtained in the making of media and on their nutritive quality is obvious. The rôle of peptone in culture media (either infusion or extract) is twofold. It furnishes nitrogenous food in the form of amino-acids and also buffer substance in the form of phosphate salts and higher nitrogen complexes (proteoses, etc.). A peptone

rich in amino-acids will, of course, furnish a basis for abundant growth. Unless, however, there is an effective buffer for regulating (suppressing) the hydrogen or hydroxyl ion, growth will be rapid at first but will soon cease. This is the experience with the Difco peptone. This peptone is richer in amino-acids (Formol titration) than Witte's peptone but is poorer in buffer (as can be seen from the character of the titration curve—small increases in acid or alkali produce appreciable changes in the P_H.) This latter condition is explicable on the basis of the high initial P_H, which may cause a precipitation of the proteoses at some stage of the preparation. This also accounts for the fact that, although richer in amino-acids, this peptone has the same total nitrogen content as Witte's peptone. It is to this difference in the content of the higher nitrogenous complexes that we ought to look for an explanation of the failure to obtain a potent diphtheria toxin with the Difco peptone.[1]

Realizing that the precipitate at P_H 8.2+ is mostly phosphates its relation to the character of the medium is quite apparent. Phosphate salts play a very important rôle in regulating bacterial metabolism and act with the proteoses as buffers in the medium. The point at which precipitation occurs is near the turning-point of phenolphthalein, the reaction of all sugar media. This explains the variability obtained in the growth of streptococci on such media, as well as the failure to obtain growth of tubercle bacilli in glycerol media made up in that way, even if the reaction is brought back to +1.0. In both cases undoubtedly the partial removal of the phosphate salt is, in itself, a factor. In the former, however, the suppression of the buffer action may be equally important.

[1] The accepted view is that diphtheria toxin is a secretion by the cell of a complex built up by it. This conception it seems to me is not supported by the facts. The more plausible idea is that it is an excretion of a residue in the cell metabolism just as ammonia or lactic acid is a waste by-product. This residue is only produced from the higher peptone complexes. That is why the Difco peptone, poor in these compounds, is not favorable for toxin production. This view also accounts for the failure on the part of *Corynebacterium diphtheriae* to produce toxin in sugar media. The sparing action of sugar on protein or rather nitrogenous metabolism is well-known.

OBSERVATIONS ON THE TYPES OF ORGANISMS ISOLATED FROM WATER AFTER TREATMENT WITH CALCIUM HYPOCHLORITE

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The hypochlorite treatment of public water supplies has within the past few years received a decided impetus. As early as 1888 the late Thomas M. Drown recommended its use, but not until 1908 did it receive serious consideration (Harrington, 1911).

The method generally employed at the present time is the addition of chlorinated lime, popularly known as "chloride of lime" or "bleaching powder," in sufficient amounts to destroy harmful bacteria and render the water potable, but not to interfere with its palatability.

The amount added is expressed in terms of "available chlorine," as determined by the usual thiosulphate titration. In reality this represents the oxygen liberated by chlorine. The reaction is expressed as follows:

$$Ca\begin{matrix}O\,Cl\\ \\ Cl\end{matrix} + CO_2 = Ca\,CO_3 + Cl_2$$

$$2\,Cl + H_2O = 2\,H\,Cl + O$$

The chlorine liberated unites with the hydrogen of the water to form hydrochloric acid, thus liberating the oxygen which accomplishes the destruction of the bacteria.

According to Clark and Gage (Rosenau, 1916) the water content before treatment is an important factor in determining the amount of chlorinated lime necessary. They found that 0.1

part of available chlorine per 100,000 parts of water effected a satisfactory purification of the Merrimac River with results equal to those of slow sand filtration. *B. coli* was entirely eliminated. In Pittsburgh it was found that 0.13 parts of chlorine per 1,000,000 parts of water were sufficient to sterilize the Alleghany River water after it had passed through the sand filters. It required, however, as much as 1 part per 1,000,000 to accomplish the same results in the raw water.

The following study was made, under the direction of Prof. C.-E. A. Winslow, in order to determine the nature of the bacteria resisting the action of chlorine in such quantities. Croton water, as supplied to New York City, is purified by the addition of calcium hypochlorite in the proportion of 1 part of available chlorin to 2,000,000 parts of water. It was decided to use this water for the experiments. On learning, however, that after treatment it passed through an open reservoir before reaching the lower end of Manhattan where the tests were made, and consequently might receive a fresh quota of saprophytic organisms, it was decided to subject it for a second time to the same treatment.

Accordingly, 3 grams of calcium hypochlorite were added to 100 liters of water (1 part available chlorine to 2,000,000 parts of water). The flasks were tightly corked, thoroughly shaken and allowed to stand two hours. At the end of that time six agar poured plates were made, each inoculated with 1 cc. of the treated water. The plates were kept for forty-eight hours at 20°C. At the end of that time one hundred and five colonies were fished. Those of most diverse appearance were selected, although, as will appear later, the majority belonged to a single group.

The cultures were examined for:

Gelatin liquefaction. The amount of gelatin liquefaction was determined in the following manner: 3 cc. of 10 per cent gelatin was placed in tubes 12 mm. in diameter, solidified, and smeared on the top with a small loop of the culture to be tested. At the end of thirty days the depth of liquefaction was measured and recorded in millimeters.

Chromogenesis. The agar cultures used to determine chromogenesis were kept at 20°C. for two weeks. A fixed portion of the growth was removed with a loop and spread evenly on white filter paper. After drying at room temperature, the color was compared with the Winslow color chart (Winslow, 1908).

Acid production in glucose peptone water. For this test Chester's (1904) method was used. Tubes containing 1 per cent glucose peptone water, made 0.8 per cent acid to phenolphthalein, were inoculated and at the end of ten days were titrated.

Bactericidal action of gentian violet. According to Churchman's method, an ordinary Petri dish was divided into two compartments by the insertion of a metal strip a little longer than the diameter of the plate. Plain agar was poured into one compartment and into the other agar plus gentian violet (dilution 1 to 100,000). In making the stroke inoculation, a loop of the broth culture was gently rubbed back and forth a number of times on the agar. The platinum needle was then sterilized and a second loop similarly applied to the gentian violet side.

Tests were also made for acid production and coagulation in litmus milk, indol production, the reduction of nitrates to nitrites, morphology and spore formation.

The following table gives the numerical results obtained:

	MORPHOLOGY		SPORE FORMATION		GELATIN LIQUEFACTION		REACTION IN LITMUS MILK	
	Bacilli	Cocci	Positive	Negative	Positive	Negative	Positive	Negative
Number of strains	100	5	89	16	68	37	98	7
Per cent.....	95.2	4.7	84.7	15.2	64.7	35.2	93.3	6.6

	INDOL PRODUCTION		ACID PRODUCTION IN GLUCOSE		REDUCTION OF NITRATES		INHIBITION BY GENTIAN VIOLET	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Number of strains	75	30	61	44	40	65	98	7
Per cent.....	71.4	28.5	58	41.9	38	61.9	93.3	6.6

It is of interest to note that none of the cocci were affected by the gentian violet, while the growth of all the bacilli, save two, was inhibited.

The organisms readily arranged themselves into five groups. *B. subtilis* far outnumbered all the others, sixty-six (62.8 per cent) belonging to that group alone.

Group 1. Rods with endospores—gelatin liquefied, 66 organisms.

Group 2. Rods with endospores—gelatin not liquefied, 23 organisms.

Group 3. Rods without endospores—gelatin not liquefied, 9 organisms.

Group 4. Rods without endospores—gelatin liquefied, 2 organisms.

Group 5. Cocci, without endospores—gelatin not liquefied, 5 organisms.

Four of the groups showed distinct subdivisions. The chart on the following page shows the relationship of the various members.

One organism (group 5–C) reacted in every way identically with those classified as *M. luteus* except that in peptone cultures it gave a decided indol reaction. According to Winslow, this property is very rare. Chester describes three coccus forms possessing the power of indol production in a slight degree, but they are also gelatin liquefiers.

CONCLUSIONS

The organisms found were apparently of the common saprophytic type usually found in air and water. No intestinal forms appeared to survive the treatment in the amount examined. It would appear, therefore, that available chlorine in the proportion of 1 part to 2,000,000 is sufficient to purify surface water obtained under conditions similar to that of the Croton supply.

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GROUP		MORPHOLOGY	SPORES	GELATIN STAB	AMOUNT OF GELATIN LIQUEFACTION	REACTION IN LITMUS MILK	INDOL	ACID PRODUCTION FROM GLUCOSE	REDUCTION OF NITRATES	INHIBITION BY GENTIAN VIOLET	CHROMOGENESIS	NUMBER OF ORGANISMS	PROBABLY IDENTICAL WITH
					mm.								
1	A	Bacilli	+	Filiform	5–20	Coagulated	+	+	+	+	Cadmium yellow to orange	36	*B. subtilis*—Cohn
	B	Bacilli	+	Filiform	10	Coagulated after 10 days	–	–	+	+	Cadmium yellow	2	*B. simplex*—Chester (1904)
	C	Bacilli	+	Filiform	10–20	Coagulated	+	+	–	+	Cadmium yellow to orange	15	*B. tumescens*—Chester
	D	Bacilli	+	Filiform	5–12	Coagulated	–	–	–	+	Cadmium yellow to orange	13	*B. ruminatus*—Chester
2	A	Bacilli	+	Filiform	–	Coagulated after 10 days	+	+	–	+	Cadmium yellow to orange	21	*B. cuticularis*—Tatoroff
	B	Bacilli	+	Filiform	–	Alkaline not coagulated	–	–	–	–	Light yellow	1	*B. ginglymus*—Ravenel
	C	Bacilli	+	Filiform	–	Alkaline not coagulated	–	–	+	–	Orange yellow	1	*B. cinctus*—Ravenel
3	A	Bacilli	–	Filiform	–	Coagulated after 10 days	–	–	–	+	Medium rose	3	*B. rubescens*
	B	Bacilli	–	Filiform	–	Coagulated after 10 days	+	–	–	+	Light cadmium yellow	6	*B. subflavus*
4		Bacilli	–	Filiform	10	No change	–	–	–	+	Light cadmium yellow	2	*B. convolutum*—Wright
5	A	Coccus	–	Beaded	–	No change	–	–	+	–	Cadmium orange	1	*Staphylococcus mollis*—Dyar
	B	Cocci	–	Beaded	–	Acid not coagulated	–	–	–	–	Light yellow	3	*M. luteus*
	C	Coccus	–	Beaded	–	Acid not coagulated	+	–	–	–	Light yellow	1	*M. luteus?*

THE MORPHOLOGY OF A STRAIN OF B. DIPHTHERIAE

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Those engaged in the production of diphtheria antitoxin have for several years been confronted with considerable difficulty in producing potent toxins. In my own experience the potency abruptly diminished about five years ago, and during subsequent years decreased in such measure as to become alarming. Intermittently, without known cause, potent toxins again resulted, but could not be produced with the same regularity as formerly, and this in spite of the fact that the organisms seemed to grow well, if film formation is taken as indication of successful proliferation.

The strain I have worked with, and which I believe is the one most commonly used, is known as Park no. 8. This strain readily forms a film on beef, veal or horse meat broth, but the nature of the film varies greatly, and in many cases those who have had experience in this line of work can foretell from the nature of the film whether a potent toxin is formed or not. Sometimes a heavy film that is thick, rough and of yellowish-brown color forms, and in these cases toxin formation is usually low. On the other hand, a friable film of a greyish tint and frequently not as heavy as the first-mentioned kind will form a potent toxin.

In casting about for a reasonable cause of the great differences in toxin production of the same strain when grown in broths made apparently alike, I have prepared many stains, primarily with the object of detecting contaminations. I was much surprised to find in many stains that the typical bacillary forms of diphtheria bacilli had disappeared and that coccus forms appeared in their place. The cocci did not appear as well-rounded forms resembling

typical staphylococci, but might easily be mistaken for streptococci in diplococcus form or in short chains. Intermediate forms were also frequently observed. These still retained the outline of a bacillus that seemed to be swollen, then broken up into cocci of various shape and size. Not infrequently diplococci reminding one of the meningococcus were seen.

That these coccus forms were variations of the original bacillary sypes was easily proved. From Löffler's blood serum, made from horse's blood, typical diphtheria bacilli always appeared. Club-shaped forms, granular bacilli, solidly stained fine bacilli in typical parallel arrangement could always be observed. By transferring the blood serum culture to a medium made of veal broth with addition of 0.2 per cent glucose and 1.5 per cent agar a luxurious growth appeared, but the stain showed the coccus forms described. When from a veal-glucose-agar culture the growth was transferred back to blood serum the original bacillary form reappeared after twenty-four hours incubation. After plating from a veal broth culture on veal-glucose-agar the colonies were all formed of diphtheria bacilli, no contamination being present. Transfers to slanted veal-glucose-agar invariably produced the coccus form, while transfers from these slants or from colonies from the plates to blood serum produced the typical bacillary form.

Since this manuscript was written a paper by Mellon (1917) came to my attention. In this paper the author describes the appearance of coccus forms of diphtheroid bacilli which he studied. The reproductions in this paper show forms exactly similar to those which I have observed. However, the author states that his diphtheroid bacilli produced a toxin distinctly different from the toxin produced by true diphtheria bacilli. My coccus-like growths produced toxins that were identical with the true diphtheria toxin, although of relatively low potency. These toxins have been used successfully for the production of diphtheria antitoxin in horses and have produced typical lesions in guinea pigs.

The observations which I have briefly described and those recorded by Mellon seem to indicate a close morphologic rela-

tionship between the members of the *B. diphtheriae* group. An exhaustive study of this relationship might lead to important scientific results and might be of practical value for the detection of diphtheria carriers, inasmuch as abnormal forms which at present lead to negative diagnoses might, when properly interpreted, show the presence of virulent diphtheria bacilli in unsuspected cases.

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MELLON, RALPH R. 1917. A study of the diphtheroid group of organisms with special reference to their relation to the streptococci. Jour. Bact., **2**, 81–107.

NOTE ON THE PREPARATION OF HYPHOMYCETES FOR MICROSCOPICAL EXAMINATION

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While excellent preparations of bacteria can be made by the familiar smear methods, every one who has worked with hyphomycetes realizes that they do not readily yield themselves to the same treatment. The densely felted mycelium does not smear well; only a few fragments remain on the slide; the fruit heads, if present, are ruptured, and the spores liberated. As a result the most satisfactory preparations of hyphomycetes obtainable by the smear method consist of a few hyphal shreads over which are scattered and lodged the liberated spores. The final preparation has but little value for a demonstration of morphological structures and relationships. This is clearly shown in the accompanying photomicrograph of a smear from a colony of *Sporotrichum* (fig. 1). As a result morphological observations are at present largely made from colonies in hanging drops, which from their nature can be but temporary preparations.

In working with a few species of the dermal hyphomycetes, it appeared as if these difficulties might be avoided if agar colonies were sectioned instead of being smeared. Typical surface colonies of a convenient size were removed from Petri dish agar cultures. In the process of removal a sharpened wire was drawn around the colony a short distance from the longest radiating mycelial threads, cutting through the entire thickness of the agar. A wire was then introduced into the agar block from the side, and it, together with the colony, removed from the Petri dish and placed in 10 per cent formalin for fixation.

After twenty-four hours fixation the colonies were removed for imbedding in celloidin in the same manner as blocks of tissue,

namely transferring successively after twenty-four hours immersion in each, from 60 per cent to 95 per cent to 100 per cent alcohol, to absolute alcohol and ether, to thin celloidin and to thick celloidin, after which the agar block containing the colony was mounted and sectioned. We have found that

FIG. 1. SMEAR FROM SPOROTRICHUM COLONY. ZEISS OBJ. 2 MM.

hematoxylin gives a more satisfactory stain than the stains ordinarily used for hyphomycetes in smear preparations. After staining the sections were decolorized, dehydrated, cleared and mounted in xylol. Colonies have been sectioned in either horizontal or vertical planes.

In preparations of this character the morphology of the various structures can be studied in their natural relationship to each other. The hyphal felting may be dense in the central portion of the colony, but in the periphery will be found radiating hyphae lying well separated, all sporangia being uninjured.

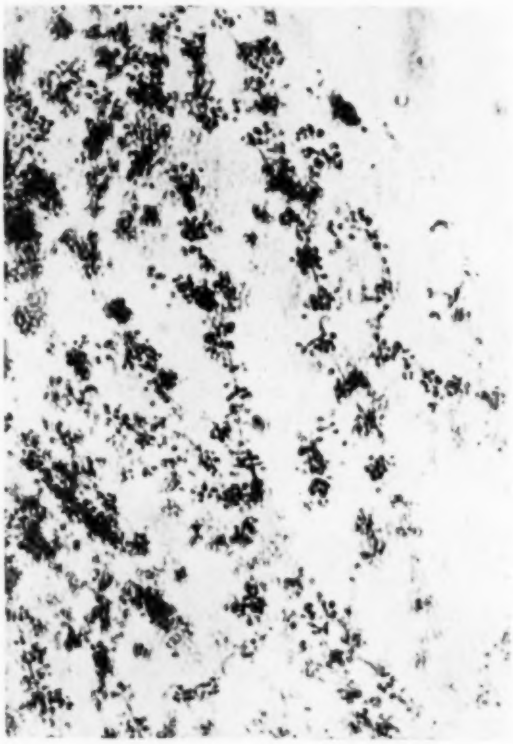

FIG. 2. SECTION OF SPOROTRICHUM COLONY. ZEISS OBJ. 4 MM.

Furthermore, the preparations are permanent mounts. Figure 2 shows a photomicrograph of a field in a vertical section of an agar colony of *Sporotrichum*. In this instance it is to be noted that the method has preserved the natural grouping of the sporangia about the hyphal threads, and that the morphology is as

clearly discernible as in a hanging drop preparation. The distorted wreckage of the smear preparation is wholly avoided.

The photomicrograhs were made by Mr. Johannes Anderson, technician of the department.

BLASTOCYSTIS HOMINIS: ITS CHARACTERISTICS AND ITS PREVALENCE IN INTESTINAL CONTENT AND FECES IN SOUTH CAROLINA

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Alexeieff (1911) gave the name *Blastocystis enterocola* to a peculiar cell found in the intestines of certain vertebrates (man, guinea pig, rat, chicken and frog) and in the snail (*Haemopsis sanguisuga*), a structure which had previously drawn the attention of numerous observers. He classified with the Ascomycetes.

Ucke (1907), Bohne and Prowazek (1908), and Bensen (1910) had described this cell as an encystment of *Trichomonas intestinalis*.

Alexeieff's contention that it could not be an encysted trichomonas but was in reality a vegetable cell was based on the mucilaginous envelope similar to the capsule of certain Blastomycetes, the observed process of budding, the presence of germinating spores, and the occurrence of the same special structure in the seeds of a certain yeast (*Schizosaccharomyces actosporus*).

Brumpt (1912), who gave the name *Blastocystis hominis* to the species found in man, and Wenyon (1915) agree with Alexeieff as to its nature.

With the view that this cell cannot be a cyst of *Trichomonas intestinalis* I am in full accord. I have seen it often in human feces where no trichomonas could be found and also in the non-trichomonas-infected intestine of man and of the rat, from examination at autopsy of bodies which had not been dead long

enough to allow the trichomonas to disappear had it been present. Then too the true cyst of *Trichomonas intestinalis* which I have described (Lynch, 1916) bears no resemblance to *Blastocystis enterocola.*

Alexeieff has studied the organism in man, the rat, frog and a snail. Wenyon has found it to be very common in soldiers returning from Gallipoli to England.

As to its presence in the United States I have been unable to find any record. It is not mentioned in various books dealing with the examination of feces in this country. I am unable to find any reference to its occurrence in our various indices to medical literature. Siler and Nichols (1911) make no mention of it in their reports of examinations of the feces of one hundred and thirteen pellagrins and two hundred and sixty-nine patients ("practically all non-pellagrous") at Peoria, Kankakee, and Dunning, Illinois. Roberts (1913) in summarizing various reports of intestinal parasites found in pellagra in America, Italy and Egypt, does not mention it. Ridlon (1916) does not record any observation of it in his study of the feces of ninety-five pellagrins in Savannah. Stiles (1915) records no observation of it in an examination of twelve hundred and eighty-seven school children for animal intestinal parasites. Rosenberger (1911) makes no mention of it in his examination of twelve hundred and eighty patients for intestinal parasites in Philadelphia, although he very kindly aided me in my first recognition of the organism and assures me that he has seen it a number of times.

Of course reports of special studies of the prevalence of animal parasites may not be expected to record the occurrence of this organism which is regarded as a vegetable cell, but from my experience in South Carolina I feel sure that it is fairly common in this country, and that the general lack of any record of its occurrence is largely due to failure to recognize it.

In the examination of forty-five pellagrins in Charleston, I have encountered the organism in twenty-five cases, or in 55.5 per cent; in company with *Endamoeba coli* in thirteen of these, and with *Trichomonas intestinalis* in eleven, while all three of

these parasites were present in six of the cases. From the examination of the feces of one hundred and sixty-eight non-pellagrous patients in the same hospital, I found sixty-eight, or 40.4 per cent, to harbor the organism, in company with *Endamoeba coli* in thirty-two, with *Trichomonas intestinalis* in twenty, and associated with both of these protozoa in sixteen of the cases.

From a similar study of the actively pellagrous inmates of the South Carolina State Hospital for the Insane at Columbia, I found the organism present in seven of twenty patients, or 35 per cent, while in the old pellagrins without symptoms of the disease it occurred in three of twenty-seven cases, or in 11.1 per cent.

In the intestine of twenty-five cases of pellagra autopsied while the body was still warm in the Roper Hospital of Charleston the organism was constantly present.

In examination of feces it has occurred in greater numbers and of larger size in those with diarrhoea, and while it has been found in formed stools it occurs in larger numbers when a purge is given to those without diarrhoea. In the postmortem examinations it has been found to be limited to the large intestine and to the cecum where it is present in large numbers; when the pellagrous inflammation has extended into the lower end of the ileum the Blastocystis has accompanied it, but has gone no further. In the intestines the number of these organisms has appeared to vary directly with the grade of inflammation present. Control examinations of the intestine of the non-pellagrous have shown it to be present in about the same proportion as in feces, and also to be limited to the large intestine.

In feces the cell conforms very well to the characteristic appearance as described by Alexeieff and by Wenyon. It varies in size from about 5 to 15 microns and is generally rounded, though elongated forms are seen. It has a delicate capsule, which apparently allows some alteration in shape, enclosing a rim of cytoplasm which may be uniform in thickness in the rounded cells or thin in the midline and broad at each end in the elongated cells. A large central body or vacuole fills the major

part of the cell. Wenyon describes the rim of cytoplasm as being of greenish appearance and containing nuclei of the appearance of greenish refractive spots, and says that the great part of the content is a large vacuole. While this appearance is commonly observed, it is just as common to find the rim of cytoplasm clear or transparent, containing highly refractive nuclear bodies, and the major portion composed of a large central body of pale hyaline greenish yellow appearance. In other words the organism apparently reverses the materials of the content of its two internal parts (see figs. 1, 2, 3 and 4). This phenomenon has been observed to occur in the course of a few minutes. A cell with the hyaline greenish yellow rim and cen-

FIGS. 1, 2, 3, 4. FRESH UNSTAINED SPECIMENS OF BLASTOCYSTIS HOMINIS FROM HUMAN FECES. COMMON FORMS SHOWING REVERSAL OF APPEARANCE OF INTERIOR

tral transparent body changed slowly to one with a transparent rim and a central body of the same appearance as was the rim previously.

In addition to this change, which may be from either form to the other, the cell has been observed to change shape (see fig. 5), passing from a rounded to an elongate form and also becoming curved while being watched for a short while. The change in shape does not appear to alter the internal characteristics.

Large rounded forms have also been seen to extrude through the capsule a large sac of transparent material, the wall and content of which was continuous with that of the central vacuole (see figs. 6, 7, 8 and 9). This sac was projected at the midline

on one side where the cytoplasmic rim was thin. It had a broad neck at first, but this gradually became constricted until the sac was turned loose and the cell became closed again. The extruded sac soon faded from view. The extrusion was as rapid as the throwing out of material from an encysting endamoeba, but the constriction and cutting loose of the sac was very gradual.

These phenomena, the reversal of internal appearances, the change in shape, and the extrusion of material from the central vacuole, have been observed only in the very fresh specimens which were kept at body temperature.

Binary division has been observed by keeping a cover glass preparation at body temperature for several days The cell divided equally until two and four were arranged in rectangular group, and then these disappeared. Stages of this division may be seen in fresh specimens from feces and intestine. Elongated forms, constricting forms and rectangular groups of two, four (see figs. 10, 11, 12 and 13) and sometimes eight cells are not uncommon.

These various phenomena have been observed chiefly in the stools of patients with diarrhoea or in specimens taken directly from the upper large intestine. In formed stools and in older specimens the small round type is all that is usually seen.

It has been interesting to observe the increase in size as well as in number from dysenteric patients, and particularly from the inflamed colon of pellagra. In formed stools from apparently normal intestines the number is small and the cells average about 8 or 9 microns in diameter. In dysenteric stools the number may be enormous and the size is larger, the cell diameter usually being around 15 microns, although many small forms may be present. In the inflamed cecum of pellagra the number is commonly large and the cells vary in size from 5 to 25 or more microns, many being very large. Especially is this true when they are present in ulcers. The exact meaning of this cannot, of course, be definitely stated until we know the properties of the organism. It appears that, while it does not propagate in the normal intestine and its contents to such an extent as to produce large numbers in feces, certain morbid processes of the large

FIG. 5. CHANGE IN SHAPE OF BLASTOCYSTIS HOMINIS IN HUMAN FECES. FRESH SPECIMEN

6 7 8 9

FIGS. 6, 7, 8 AND 9. EXTRUSION OF MATERIAL FROM CENTRAL VACUOLE BY BLASTOCYSTIS HOMINIS IN HUMAN FECES

10 11 12

FIGS. 10, 11 AND 12. FRESH UNSTAINED SPECIMENS OF BLASTOCYSTIS HOMINIS FROM HUMAN FECES. ELONGATING FORMS

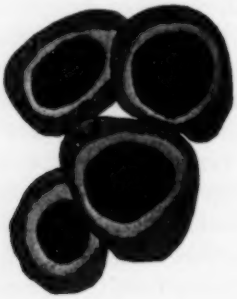

FIG. 13. GROUP OF BLASTOCYSTIS HOMINIS FROM CECUM IN CASE OF PELLAGRA AT AUTOPSY. HEIDENHAIN'S IRON-HAEMATOXYLIN AND EOSIN STAIN.

gut offer a better field, and it increases in number and also in size, while in case of diarrhoea large numbers and also many large vegetative forms may be thrown out. It appears that the pellagrous intestine is a splendid field for the growth of this cell, as it also appears to be for small yeasts, since they are usually abundant. The organism is not so common and is of smaller size in the feces of old pellagrins in whom the symptoms of the disease have disappeared.

It has also been interesting to note the differences in viability of the organism from different sources. In all specimens it has disappeared in the course of a few days, and in some exposure for a few hours leads to its elimination. It seems that in the formed stool, where the small rounded form is present, it lasts longest, while in the diarrhoeal stool or in the specimen from the inflamed cecum, where large cells showing the various active phenomena previously described are seen, it does not survive long. Apparently this small rounded type is the resistant stage of the organism, and the large active forms are vegetative non-resistant stages. While examples of binary division are commonly observed, I have not seen the process of budding described by Alexeieff.

As to the liability of the uninitiated to confuse *Blastocystis enterocola* with cysts of the endamoebae and trichomonas, I must agree with other writers, since at one time I looked upon it and described it as probably a cyst of *Trichomonas intestinalis*. However, there are distinct points of difference which if known cannot fail to clear up the difficulty. The typical pear shape, the nearly constant size (about 6 by 8 microns), the nucleus and the peculiar internal structures of the cyst of *Trichomonas intestinalis*, should make the recognition of this body not difficult. As between endamoeba and Blastocystis, the cysts of the former are also of fairly constant size, are round, have a thick capsule, the body being uniform and the nuclei, especially in stained specimens, characteristic; while the Blastocystis may vary from 5 to 20 or more microns in diameter in the same preparation, especially when diarrhoea is present or a purgative has been given, it may show rounded and elongated forms, and

may be single or in rectangular groups of two, four or eight. It also has a thin flimsy capsule, and a body composed of two parts, a cytoplasmic rim which may be transparent or of a pale hyaline greenish yellow, uniform in thickness in the rounded cells, but thin in the midline and thicker at the poles in the elongated cells, containing the highly refractive nuclear bodies commonly in groups in the broad portion at the poles, and a large central body which may be transparent or of pale hyaline greenish yellow.

In specimens stained with Leishman's modification of Wright's method the Blastocystis either has a bluish central body and pink cytoplasmic rim with reddish nuclear bodies or a pink central body and a bluish rim with red nuclear bodies, depending on the stage of the cell. It takes the Heidenhain iron-haematoxylin stain very well (see fig. 10), the rim staining pink, when counterstained with eosin, with black nuclear bodies and the central body appearing as a dark smoky or black body within a large clear vacuole. Apparently in the fixing process, this central body shrinks away from its wall leaving an empty space.

As to the exact nature of the cell, I feel that there is more to be learned. It is true that it bears strong resemblance to yeasts. I have, however, been unable to cultivate the organism in various media including those specially designed for the growth of yeasts, and, in so far as I have been able to learn, this has not been accomplished. Then too, the reversal of the appearance of the materials of the central body and cytoplasmic rim, and the extrusion of material from the central vacuole, are phenomena that I am unacquainted with in the yeasts; and for a yeast body it is singularly non-resistant, especially in the large active form.

As to the properties of *Blastocystis enterocola*, other than its morphology, we know nothing. Therefore, the full significance of its presence cannot be judged. I have seen it in the intestine of rats, and it has presented itself to me as a common intestinal parasite of man to which no attention has been paid in this country. Because of the liability of the observer to confuse it

with other encysted intestinal parasites, and since it has appeared to me to be possibly comparable to endamoeba, trichomonas, lamblia, etc., as an indicator of the contamination of the food or drink of an individual or a community with fecal matter of man or of certain lower animals, I consider it of sufficient importance to warrant a report of these observations.

As an intestinal parasite, of the importance of which we know little or nothing, it should receive our serious consideration.

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CHARACTERISTICS OF COLI-LIKE MICROORGANISMS FROM THE SOIL

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I. ISOLATION OF ORGANISMS AND PRELIMINARY OBSERVATIONS

It has been generally believed that all of the aerobic, non-spore forming, lactose-fermenting bacteria are essentially of fecal origin. Prescott and Papasotiriu found similar organisms on grains, but it appears from the very careful and painstaking studies of Rogers and his associates of the United States Department of Agriculture that the grain types may be differentiated from the fecal organisms. They demonstrated that the grain strains decomposed glucose with the liberation of two or more times as much CO_2 as H_2, whereas the bovine fecal strains formed approximately equal volumes of these gases. Later Clark and Lubs showed that in appropriate glucose media the former were alkaline and the latter acid to methyl red. Levine, employing the methyl red and Voges-Proskauer reactions, observed that an alkaline methyl red reaction was correlated with a positive Voges-Proskauer test and that coli-like organisms giving these reactions were not uncommon in sewage, but were presumably absent, or at least very rarely present, in the feces of man, horse, sheep and pig as well as the cow.

The occurrence of these methyl-red negative, Voges-Proskauer positive organisms in sewage coupled with their absence in feces, and the observation of Clemesha, in India, that the incidence of *B. lactis-aerogenes* (a Voges-Proskauer positive organism) increases considerably in lakes and rivers after rainfall, naturally led to the inference that they are essentially soil forms.

Of the previous works on the coli-like bacteria of the soil, those of Houston in England and Konrich in Germany are probably of widest sanitary significance.

Houston examined a number of samples of orchard, garden, pasture and virgin soils including both polluted and unpolluted areas. He concludes that *B. coli* or its "close allies" are very rarely or never found in virgin soils, while they are present in large numbers in other soils especially those which have been contaminated from animal sources.

Konrich examined 547 samples of soil in Germany; 65 per cent showed *B. coli* in from 0.1 to 0.5 gram. He notes that the further away the source was from cultivation the smaller was the proportion of positive results.

In neither of these studies were the organisms isolated differentiated from fecal strains.

The present investigation was undertaken to determine (1) if the methyl-red negative or Voges-Proskauer positive organisms are the predominant coli-like forms in soil, and (2) to study the characteristics of the various types of aerobic lactose-fermenting organisms isolated from soils.

Source of samples. Forty-two samples were studied, including 9 from different parts of a corn field and one from a clover field in Ames, Iowa; 13 from fallow and 11 from cropped experimental plots; 4 from orchards; and 4 miscellaneous samples sent in by the Soils Department from different parts of the state.

Collection of samples. All samples were secured in October and the early part of November, 1915. The eight orchard and miscellaneous samples furnished by the Soils Department were parts of sample regularly collected for chemical analysis. All other samples consisted exclusively of surface earth collected in the following manner. A sterile wide-mouthed bottle was held horizontally upon the ground, the stopper carefully removed, and 50 to 75 grams of the surface soil (usually from the first inch) was scraped into the bottle with a sterile metallic scalpel or spatula. The bottle was then closed securely, labeled, and taken to the laboratory where it was examined within two or three hours after collection. The spatula or scalpel was sterilized, between samples, by dipping in alcohol and flaming.

Technique. Immediately on arrival at the laboratory, the soil was transferred to a liter flask of sterile water, thoroughly shaken, and after standing for fifteen minutes, or long enough to permit sedimentation of the heavier soil particles, various dilutions of the supernatant liquid were plated on litmus lactose agar. Incubation was at 37°C. One cubic centimeter was also inoculated into an anaerobic Durham tube of lactose broth sealed with paraffine oil. If gas was formed after twenty-four to forty-eight hours at the body temperature litmus lactose agar plates were poured. From the plates made directly or after preliminary enrichment, acid colonies were fished into standard lactose and sucrose broth and into 0.5 per cent glucose-peptone-dipotassium-phosphate solution, in Durham fermentation tubes. After three days at 37°C. gas was recorded and also the methyl-red and Voges-Proskauer reactions. For the methyl-red reaction 0.2 cc. of the indicator was added to 5 cc. of the glucose culture. The Voges-Proskauer reaction was determined by adding an equal volume of 10 per cent KOH to 2 or 3 cc. of the glucose medium. After standing six to twenty-four hours an eosine-like coloration developed if the test was positive.

Results of preliminary studies

The results may be conveniently considered on the basis of the source of the samples under the following heads. Samples from (1) corn fields; (2) fallow experimental plots; (3) cropped experimental plots; (4) miscellaneous samples about which no definite or detailed information was available.

Samples from corn fields. Samples A, B, C, D, E, and H were taken from widely separated parts of a corn field while the crop was still standing. Samples XXXIV and XXXV were obtained from the same field six weeks later; the corn was already in shock. Corn had been grown on this field for three years and manure was last applied in 1913 or two years before this study. One sample, XXXVI, was obtained from a field of Kafir corn, but no information as to its soil treatment was obtained.

Lactose-fermenting bacteria were obtained from eight of these nine samples. Sixty-six coli-like organisms were isolated. Of these 61 (92.5 per cent) formed gas from sucrose, 43 (65.2 per cent) were positive for the Voges-Proskauer reaction, while 56 (84.9 per cent) were alkaline to methyl red.

Samples from fallow experimental plots. Thirteen samples (I to XIII) were obtained from fallow experimental plots. These plots had been under the supervision of the Soils Department of the Iowa State College for seven years during which period they were free from weeds and kept absolutely fallow. Data as to their treatment is indicated in table 1. Samples were taken prior to the 1915 treatment.

Coli-like organisms were not obtained from seven samples I, II, III, IV, VI, VII, and IX. Samples VIII and X each yielded a single lactose fermenter. From samples V, XI, XII and XIII, 83 coli-like cultures were obtained.

Of the 85 organisms isolated, 84 (98.8 per cent) formed gas from lactose and 57 (67.1 per cent) from sucrose; 53 (62.3 per cent) were positive for the Voges-Proskauer reaction, and 53 (62.3 per cent) were alkaline to methyl red.

Samples from cropped experimental plots. Eleven samples were taken from experimental plots on which crops were growing. These plots were also under observation of the Soils Department for seven years previous. On plots of samples XIV and XXXII timothy was grown each year and the stubble plowed under. No other treatment was given. The other nine samples XXIII–XXXI) were treated in various ways as indicated in table 1, and corn was raised upon them each year. Samples were collected in October, 1915, while the crops were still standing.

Coli-like organisms were isolated from every sample. Of 166 cultures obtained, 158 (95.2 per cent) gave gas from lactose and 143 (86.2 per cent) from sucrose; 97 (58.4 per cent) were positive for the Voges-Proskauer reaction and 199 (71.8 per cent) alkaline to methyl red.

Miscellaneous samples. In this group are included 9 samples about which no information as to soil treatment was available.

TABLE 1

Source, treatment, etc., of 42 samples of soil studied

SAMPLE	SOURCE	SOIL TREATMENT OR OTHER REMARKS	DATE OF LAST TREATMENT	CROP	NUMBER OF COLI-LIKE ORGANISMS OBTAINED
A	Corn field	Manured, corn grown for three years	1913	Corn	0
B	Corn field	Manured, corn grown for three years	1913	Corn	14
C	Corn field	Manured, corn grown for three years	1913	Corn	4
D	Corn field	Manured, corn grown for three years	1913	Corn	3
E	Corn field	Manured, corn grown for three years	1913	Corn	2
H	Corn field	Manured, corn grown for three years	1913	Corn	9
I	Expt. plot 114	4 tons manure annually	1914	None	0
II	Expt. plot 113	2 tons manure annually	1914	None	0
III	Expt. plot 112	1 ton manure annually	1914	None	0
IV	Expt. plot 111	4 tons of clover chopped and ploughed under annually	1914	None	0
V	Expt. plot 110	2 tons of clover chopped and ploughed under annually	1914	None	18
VI	Expt. plot 109	1 ton of clover as in IV	1914	None	0
VII	Expt. plot 108	2 tons of oat straw chopped and ploughed under annually	1914	None	0
VII	Expt. plot 107	No treatment, check plot	1914	None	1
IX	Expt. plot 106	2 tons of timothy chopped and ploughed under annually	1914	None	0
X	Expt. plot 105	1 ton timothy as in IX	1914	None	1
XI	Expt. plot 104	8 tons of clover once in four years	1913	None	26
XII	Expt. plot 103	8 tons manure once in four years	1913	None	24
XIII	Expt. plot 102	28 tons peat annually	1914	None	14
XIV	Expt. plot 101	Timothy grown, each year and stubble plowed under	1914	Timothy	18
XV	Orchard	Cloved sod		Clover	12

TABLE 1—Continued

SAMPLE	SOURCE	SOIL TREATMENT OR OTHER REMARKS	DATE OF LAST TREATMENT	CROP	NUMBER OF COLI-LIKE ORGANISMS OBTAINED
XVI	Orchard	Cover crop planted in summer and ploughed under in spring		?	0
XVII	Orchard	Clean cultivation			0
XVIII	Orchard	Blue grass sod		Blue grass	8
XIX	Sioux loam	Clover field recently in oats		Clover	1
XX	Wabash silty clay loam	Surface soil			1
XXI	Marshall silt loam	Surface soil			8
XXII	Corrington loam	Surface soil; corn field		Corn	5
XXIII	Expt. plot 213	No treatment, check plot		Corn	13
XXIV	Expt. plot 214	Legume treatment, cow peas in July. Two years out of four in rotation with corn	1913	Corn	18
XXV	Expt. plot 215	8 tons manure	1913	Corn	15
XXVI	Expt. plot 216	8 tons manure with cow peas	1913	Corn	14
XXVII	Expt. plot 217	8 tons manure with 800 pounds bone meal and cow peas	1913	Corn	16
XXVIII	Expt. plot 218	800 pounds bone meal and cow peas	1913	Corn	16
XXIX	Expt. plot 219	8 tons manure and 800 pounds bone meal	1913	Corn	13
XXX	Expt. plot 220	800 pounds bone meal and 200 pounds K in KCl and cow peas	1913	Corn	19
XXXI	Expt. plot 221	8 tons manure, 800 pounds bone meal and 200 pounds K in KCl	1913	Corn	12
XXXII	See XIV	See XIV	1913	Timothy	10
XXXIII	Clover field	Unknown	1913	Clover	13
XXXIV	Corn field	Same as A	1913	Corn	17
XXXV	Corn field	Same as A	1913	Corn	15
XXXVI	Corn field	Unknown	1913	Kafir corn	2

Samples XVI and XVII did not yield any coli-like organisms. From XIX and XX a single culture was recovered in each case. From the five samples remaining, XV, XVIII, XXI, XXII and XXXIII, 46 coli-like organisms were obtained.

Of the 48 strains isolated, 45 (93.8 per cent) formed gas from lactose, and 43 (89.6 per cent) from sucrose; 27 (56.3 per cent) were positive for the Voges-Proskauer reaction and 35 (73 per cent) were alkaline to methyl red.

A comparison of the fallow and cropped experimental plots with respect to the presence of coli-like forms is shown in table 2. It is seen from tables 1 and 2 that, whereas coli-like bacteria

TABLE 2

Presence of coli-like bacteria in experimental soil plots at Ames, Iowa

COLI-LIKE ORGANISMS	FALLOW PLOTS		CROPPED PLOTS	
	Number	Per cent	Number	Per cent
Absent	7	53.8	0	0.0
Rare	2	15.4	0	0.0
Abundant	4	30.8	11	100.0

were only occasionally isolated from fallow plots, some of which had considerable quantities of manure applied at different times to them, the cropped experimental plots invariably showed coli-like bacteria present.

The reactions of the 365 organisms isolated are summarized in table 3. The organisms from the fallow experimental plots attacked sucrose less frequently (67.1 per cent), than those from other sources (86.2 to 92.5 per cent).

These preliminary observations indicate that among the soils studied

1. Coli-like organisms are more abundant in soils bearing crops (corn, clover and timothy) than in fallow soils, even though considerable quantities of manure had been added to the latter.

2. The most common types of coli-like forms found in soil are sucrose fermenters, alkaline to methyl red in glucose media and reacting positively to the Voges-Proskauer test.

TABLE 3

Preliminary observations of 365 coli-like bacteria obtained from soil

	Organisms from									
	Corn fields		Fallow experimental plots		Cropped experimental plots		Miscellaneous samples		All samples	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Voges-Proskauer positive	43	65.2	53	62.3	97	58.4	27	56.3	220	60.2
Methyl-red negative	56	84.9	53	62.3	119	71.8	35	73.0	263	72.1
Gas from:										
Glucose	66	100.0	85	100.0	166	100.0	48	100.0	365	100.0
Lactose	66	100.0	84	98.8	158	95.2	45	93.8	353	99.5
Sucrose	61	92.5	57	67.1	143	86.2	43	89.6	304	83.4

3. Of 365 cultures studied, all forming gas from glucose, 353 (99.5 per cent) gave gas from lactose and 304 (83.4 per cent) from sucrose; 220 (60.2 per cent) were positive for the Voges-Proskauer reaction, and 263 (72.1 per cent) were alkaline to methyl red.

4. The methyl red and Voges-Proskauer reactions seem well correlated. Of 263 alkaline to methyl red 81 per cent gave a positive Voges-Proskauer reaction, while among 220 strains which gave the Voges-Proskauer test 96 per cent were alkaline to methyl red.

II. TYPES OF COLI-LIKE BACTERIA IN THE SOIL

The preceding observations were preliminary in character and some of the cultures were probably not pure. The organisms were therefore re-purified by plating on brilliant green agar containing 1 part in 500,000 of the dye. A very small loop of a twenty-four hour lactose broth culture of the organism to be purified was placed near the center of a Petri dish containing brilliant green agar and smeared over the surface of the medium with a sterile glass rod. A second plate was then smeared with the same rod. The Petri dishes were incubated for forty-

eight hours at 37°C. The first plate often showed extensive over-growths, but in these instances well isolated colonies were almost always present on the second plate. If acid colonies were present, one was fished into lactose broth. If distinctly acid colonies were not present, then a colony which most nearly resembled *B. coli* was fished into lactose broth. The broth tubes were incubated at 37°C. for forty-eight hours and, if gas was formed, agar slants were made from them.

Upon some of the brilliant green agar plates two types of colonies were present. They were similar in shape and often in size, but one was white and heavy while the other showed a light or opalescent thin growth. Where these two kinds of colonies appeared fishings were made from both.

About 400 cultures were obtained, but as these were too many to handle only 184 were studied in detail.

Characters studied

Morphology, Gram's stain, acid production and coagulation in milk, gelatin liquefaction, indol production, motility, the methyl red reaction and production of gas and acetyl-methyl-carbinol from various substances were observed.

Morphology and gram stain. Gram stains were made from young (less than twelve hours old), agar slant cultures. Freshly prepared staining solutions were employed and their reliability determined by staining known positive (*B. subtilis*) and negative (*B. coli*) organisms. Smears were immersed for one minute in anilin oil gentian violet, then for one minute in Gram's iodine solution, decolorized for five minutes in 95 per cent alcohol and counterstained for fifteen seconds with dilute safranin. All of the organisms studied are regarded as Gram-negative, but it was observed that some were not decolorized as completely as others.

Morphology was determined from the Gram stain. Five of the cultures seemed to contain both long and short rods. All of the others (179) were short rods.

Milk. Acid production and coagulation was observed in

azolitmin milk. The reaction of the medium was + 1 to phenolphthalein and incubation was at 37°C. for forty-eight hours. Acid production was recorded as: + acid; sl. slightly acid; ± no change or neutral; − alkaline.

Coagulation after forty-eight hours incubation without heating was recorded +. Tubes which did not show coagulation were heated in a boiling water bath for one or two minutes.

Gelatin liquefaction. Stab cultures in nutrient gelatin were observed for thirty-four days at 20°C. Observations were made after 2, 7, 15, 20, 27 and 34 days. The records finally used were for the thirty-four day period.

Motility. Motility was determined from stab cultures in semi-solid nutrient or Hesse agar (5 grams agar per liter) as suggested by Kligler. The medium was kept in the 37° incubator for two or three hours before inoculation. Where motility was not present, growth was confined to the line of inoculation, the rest of the medium remaining perfectly clear. The motile forms quickly grew out away from the line of inoculation in all directions, causing a diffuse clouding in the medium. Very often the growth extended for a distance of 10 or 15 mm. in six hours at 37°C. Observations were made after six and twenty-four hours.

Indol production. The formation of indol from 1 per cent Witte's peptone solution was determined after incubation at 37°C. for four days. The test was made by adding a few drops of 10 per cent H_2SO_4 to about 8 cc. of the peptone culture, and, after shaking the mixture, 1 cc. of a freshly prepared 0.01 per cent $NaNO_2$ solution was added so as to form a layer on the surface. The tubes were set aside for five or six hours after which time the presence or absence of the nitroso-indol coloration at the junction of the two liquids was recorded.

Methyl-red reaction. For the methyl-red reaction the 0.5 per cent peptone di-potassium-phosphate-glucose medium suggested by Clark and Lubs was employed. Incubation was at 37°C. for forty-eight hours, and 0.2 cc. of the indicator was added to 5 cc. of the culture for the test.

Gas and acetyl-methyl carbinol production. The monosaccharids

glucose and galactose, the disaccharids lactose and sucrose, the trisaccharid raffinose, glucoside salicin, the alcohols glycerol, mannitol and dulcitol, and the polysaccharids dextrin, inulin and starch were studied for gas formation and acetyl-methyl-carbinol production. The medium for these reactions consisted of 0.5 per cent di-potassium phosphate peptone solution to which was added the test substance. The following quantities of test materials were employed: glucose, galactose and lactose 0.5 per cent; raffinose, dulcitol and salicin 0.71 per cent; sucrose, mannitol, glycerol, dextrin, inulin and starch 0.75 per cent.

The polysaccharids were incubated for seventy-two hours and the other test substances for forty-eight hours at 37°C.

Gas formation was observed in Durham fermentation tubes. Acetyl-methyl-carbinol was tested for by adding 10 per cent KOH solution to an equal volume of the culture and allowing the mixture to stand exposed to the air. Records were made after five or six and after twenty-four hours. The results were recorded as follows: + gas positive, acetyl-methyl carbinol positive; ± gas positive, acetyl-methyl carbinol negative; − gas negative, acetyl-methyl carbinol negative; ∓ gas negative, acetyl-methyl carbinol positive.

Correlation of results

The correlation of the Voges-Proskauer and methyl-red reactions with these purified cultures was more marked than was obtained in the preliminary tests. Of 139 which gave the Voges-Proskauer reaction 132 (95.0 per cent) were alkaline, 4 (2.9 per cent) were in the neutral tints, and 3 (2.1 per cent) acid to methyl red. Of 134 strains alkaline to methyl red 132 (98.6 per cent) gave the Voges-Proskauer reaction. Of 45 strains which did not give the Voges-Proskauer reaction after forty-eight hours at 37°C. 30 (66.7 per cent) were acid, 13 (28.9 per cent) in the neutral tints and 2 (4.4 per cent) alkaline to methyl red. The two alkaline and 8 of the 13 neutral strains attacked the polysaccharids dextrin and starch and were non-motile, while all of the other Voges-Proskauer negative strains

were motile and did not form gas from the polysaccharids. We believe that on longer incubation (five to seven days) these 8 neutral strains would have reverted to a distinct alkaline reaction, and together with the two alkaline cultures would probably react positively for the Voges-Proskauer test.

In table 4 are shown the number and per cent of positive reactions for 177 organisms which formed gas from lactose. Tests which were uniformly negative or positive are not included in the table. With 7 cultures gas formation from lactose was questionable. These are discussed later in more detail.

TABLE 4

Reactions of aerobic non-sporing lactose-fermenting bacteria in the soil

CHARACTER	NUMBER POSITIVE	PER CENT POSITIVE
Voges-Proskauer	142	80.3
Motility	123	69.6
Gelatin	83	46.8
Indol	66	37.3
Sucrose	165	93.3
Raffinose	162	91.6
Dulcitol	74	41.8
Glycerol	78	43.1
Salicin	159	89.9
Dextrin	82	46.4
Inulin	21	11.9
Starch	57	32.2

From table 4 it is seen that sucrose, raffinose and salicin are almost always attacked, with gas formation, as indicated by 93.3 per cent, 91.5 per cent and 89.9 per cent positive tests respectively. A large proportion, 80.3 per cent, gave a positive Voges-Proskauer reaction, and 69.6 per cent were motile. Gelatin liquefiers were quite common (46.8 per cent), as were also polysaccharid fermenters (dextrin 46.4 per cent, inulin 11.9 per cent and starch 32.2 per cent). Gas was formed from dulcitol by 41.8 per cent; and glycerol by 43.1 per cent. Only 37.3 per cent formed indol.

In general the reactions of the organisms isolated from soil are quite different from those obtained by Levine from various ani-

mal feces and sewage. A comparison of the reactions of the fecal and sewage strains with the soil strains is shown graphically in figure 1.

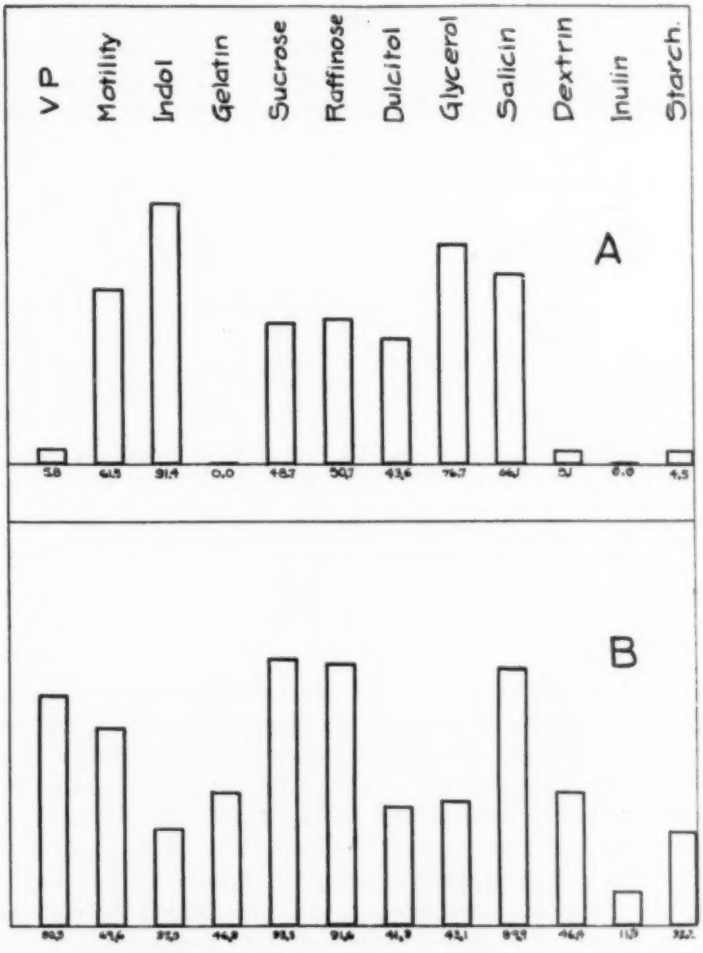

FIG. 1. PER CENT OF POSITIVE REACTIONS OF COLI-LIKE BACTERIA FROM (*A*) FECES AND SEWAGE, AND (*B*) SOIL

There are three distinct and rather well defined groups of aerobic non-spore forming lactose-fermenting bacteria in the soil. The characteristics of these groups, together with that of a small group which we do not feel justified in regarding as distinct for the present, are shown in figure 2 and table 5.

Group A (B. coli type). Thirty-five organisms are included in Group A. All are motile, Gram negative, do not form gas from starch nor liquefy gelatin, and react negatively to the Voges-

TABLE 5

Characteristics of the groups of coli-like bacteria obtained from the soil

GROUP		VOGES-PROSKAUER	MOTILITY	GELATIN	INDOL	SUCROSE	RAFFINOSE	DULCITOL	GLYCEROL	SALICIN	DEXTRIN	INULIN	STARCH
A	Number +...	0	35	0	23	26	26	32	17	18	0	0	0
	Per cent +...	0.0	100.0	0.0	65.6	74.3	74.3	91.5	48.6	51.5	0.0	0.0	0.0
B'	Number +...	0	0	0	3	10	10	2	10	10	10	5	10
	Per cent +...	0.0	0.0	0.0	30.0	100.0	100.0	20.0	100.0	100.0	100.0	50.0	100.0
B	Number +...	44	0	2	22	44	44	26	43	44	44	13	43
	Per cent +...	100.0	0.0	45	50.0	100.0	100.0	59.1	97.7	100.0	100.0	29.6	97.7
C	Number +...	88	88	81	15	85	82	14	8	87	28	4	4
	Per cent +...	100.0	100.0	92.1	17.1	96.6	93.2	15.9	9.1	98.9	31.8	4.5	4.5

Proskauer test. Thirty-two are short rods and 3 are presumably mixed as they contained a few long rods as well. Acid was formed in milk by all of the strains and a coagulum was produced in forty-eight hours at 37°C., without heating. Gas was formed as follows: sucrose 74.3 per cent, raffinose 74.3 per cent, dulcitol 91.5 per cent, glycerol 48.6 per cent, and salicin 51.5 per cent. The polysaccharids, dextrin, inulin and starch were not attacked, with gas formation. Indol was formed by 65.6 per cent. In table 6 is shown the correlation of each reaction with all other reactions. Where a reaction was uniformly negative or positive it is not shown in tabular form but indicated by a

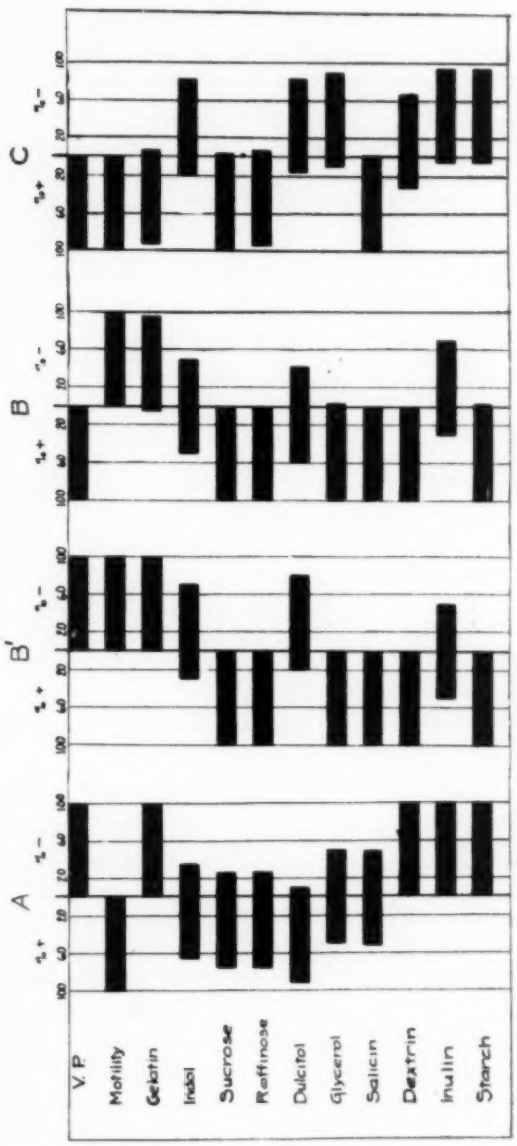

FIG. 2. REACTIONS OF MAIN GROUPS OF COLI-LIKE BACTERIA OBTAINED FROM SOIL

(*A*) *B. Coli* type; (*B* and *B'*) *B. Aerogenes* type; (*C*) *B. cloacal* type

note. It appears from this table and from chart 2 that group A is not a homogenous one, but that it may be further subdivided according to the reaction on sucrose, or perhaps, salicin or glycerol.

Groups B and B' (B. aerogenes type). Group B includes 44 organisms which give a positive Voges-Proskauer reaction, are non-motile, and practically always form gas from starch. All

TABLE 6

Correlation of reactions in group A (B. coli type) of lactose-fermenting bacteria from the soil

		INDOL		SUCROSE		RAFFINOSE		DULCITOL		GLYCEROL		SALICIN	
		+	−	+	−	+	−	+	−	+	−	+	−
Indol	+	23		19	4	18	5	23		7	16	14	9
	−		12	7	5	8	4	9	3	10	2	4	8
Sucrose	+	19	7	26		24	2	25	1	12	14	16	10
	−	4	5		9	2	7	7	2	5	4	2	7
Raffinose	+	18	8	24	2	26		25	1	13	13	15	11
	−	5	4	2	7		9	7	2	4	5	3	6
Dulcitol	+	23	9	25	7	25	7	32		14	18	18	14
	−		3	1	2	1	2		3	3			3
Glycerol	+	7	10	12	5	13	4	14	3	17		5	12
	−	16	2	14	4	13	5	18			18	13	5
Salicin	+	14	4	16	2	15	3	18		5	13	18	
	−	9	8	10	7	11	6	14	3	12	5		17

All strains are motile. Gelatin liquefaction, the Voges-Proskauer reaction, and gas formation from dextrin, inulin and starch were in all cases negative.

are gram negative short rods, but one culture also contained some long rods. With respect to the azolitmin milk reaction, it was observed that 8 (18.6 per cent) were only slightly acid after forty-eight hours at the body temperature, while 14 (31.8 per cent) were not coagulated except by heating. Gelatin was liquefied by 2 cultures (4.5 per cent). Positive tests for gas formation were observed as follows: sucrose 100 per cent; raffinose 100 per cent; dulcitol 59.1 per cent; glycerol 97.7 per cent;

salicin 100 per cent; dextrin 100 per cent; inulin (29.6 per cent) and starch 97.7 per cent. Indol was formed by 50 per cent of the strains.

In table 7 is shown the correlation of the various reactions studied. Those uniformly positive or negative are omitted. From this table and chart 2, it appears that subdivision might be made on the basis of indol, dulcitol, or inulin reactions. These reactions, however, are not correlated with each other. Of 22 organisms which formed indol 15 (68.2 per cent) gave gas from dulcitol and 7 (31.8 per cent) from inulin, whereas among an

TABLE 7

Correlation of reactions in group B (B. aerogenes type) of lactose-fermenting bacteria obtained from the soil

		INDOL		DULCITOL		INULIN	
		+	−	+	−	+	−
Indol	+	22		15	7	7	15
	−		22	11	11	6	16
Dulcitol	+	15	11	26		6	20
	−	7	11		18	7	11
Inulin	+	7	6	6	7	13	
	−	15	16	20	11		31

All strains were non-motile; 2 liquefied gelatin; 1 did not form gas from glycerol, and 1 from starch; all formed gas from sucrose, raffinose, salicin, and dextrin.

equal number of indol-negative-strains, 11 (50.0 per cent) gave gas from dulcitol and 6 (27.3 per cent) from inulin. Similarly, there does not seem to be any correlation between gas formation from dulcitol and inulin. 23.1 per cent of the dulcitol-positive and 38.8 per cent of the dulcitol-negative strains gave gas from inulin. We would, therefore, regard the group as a whole as the species *B. aerogenes*, and the sub-groups formed by splitting of inulin, indol or dulcitol as varieties.

Group B′ includes ten organisms which did not give the Voges-Proskauer reaction but which attacked the polysaccharids very readily. In these respects the group seems to be intermediate

between A and B. The reaction to methyl red was also inconclusive. Two strains were alkaline and eight in the neutral tints after forty-eight hours at 37°C. As has been previously suggested, we believe that the methyl red reaction would become distinctly alkaline on further incubation, and that the organisms would probably also give the Voges-Proskauer reaction. With respect to motility, indol production, gelatin liquefaction, and gas formation from various fermentable substances, the organisms in Group B′ are so strikingly similar to Group B, and so different from either group, A or C, that it is perhaps best to include them under the species *B. aerogenes*.

Group C (*B. cloacae type*). This group contains 88 organisms and constitutes the predominant form of coli-like microorganisms in the soil. The organisms give the Voges-Proskauer reaction, are motile, and probably always liquefy gelatin. All are Gram-negative short rods. The reaction in azolitmin milk is quite different from that observed in group A. Only 13 cultures (14.8 per cent) of group C were distinctly acid, and 75 (85.2 per cent) were but slightly acid. With 15 strains (17.1 per cent) a coagulum was obtained in the milk without heating; 68 (77.2 per cent) coagulated after heating in a boiling water bath for one or two minutes; with 5 (5.7 per cent) coagulation did not take place even after heating for ten minutes.

A large number of these strains (about 75 per cent) formed only 10 per cent or less gas from lactose. The test for gas formation from lactose was repeated several times in order to verify the fact that the organisms were lactose fermenters. The last observation was made in 1 per cent Witte's peptone containing 0.5 per cent di-potassium phosphate and 1 per cent of Difco lactose guaranteed to be free from glucose. The medium was carefully sterilized for ten minutes at 10 pounds, the pressure quickly released and the medium rapidly cooled by immersion of the tubes in cold water. A very light colored medium was obtained, and it is believed that inversion of the lactose was avoided. The records finally accepted were those made with this medium.

The reactions of group C are as follows:

Gelatin liquefied 92.1 per cent; indol 17.1 per cent; gas from sucrose 96.6 per cent; raffinose 93.2 per cent; dulcitol 15.9 per cent; glycerol 9.1 per cent; salicin 98.9 per cent; dextrin 31.8 per cent; inulin 4.5 per cent and starch 4.5 per cent.

TABLE 8

Correlation of reactions in group C (B. cloacae type) of lactose fermenting bacteria obtained from the soil

		INDOL		SUCROSE		RAFFINOSE		DULCITOL		GLYCEROL		DEXTRIN		INULIN		STARCH	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Indol	+	15		15		15		7	8	6	9	8	7	2	13	2	13
	−		73	70	3	67	6	7	66	2	71	20	53	2	71	2	71
Sucrose	+	15	70	85		81	4	13	72	8	77	28	57	4	81	4	81
	−		3		3	1	2	1	2		3		3		3		3
Raffinose	+	15	67	81	1	82		13	69	7	75	26	56	3	79	4	78
	−		6	4	2		6	1	5	1	5	2	4	1	5		6
Dulcitol	+	7	7	13	1	13	1	14		7	7	6	8	2	12	2	12
	−	8	66	72	2	69	5		74	1	73	22	52	1	73	2	72
Glycerol	+	6	2	8		7	1	7	1	8		5	3	2	6	2	6
	−	9	71	77	3	75	5	7	73		80	23	57	2	78	2	78
Dextrin	+	8	20	28		26	2	6	22	5	23	28		4	24	4	24
	−	7	53	57	3	56	4	8	52	3	57		60		60		60
Inulin	+	2	2	4		3	1	2	1	2	2	4		4		3	1
	−	13	71	81	3	79	5	12	73	6	78	24	60		84	1	83
Starch	+	2	2	4		4		2	2	2	2	4		3	1	4	
	−	13	71	81	3	78	6	12	72	6	78	24	60	1	83		84

All strains are motile and form gas from salicin. Gelatin not liquefied in thirty-four days by 7 strains.

Table 8 shows the correlation of the various reactions. Reference to this table and figure 2 indicates that the group is a rather homogenous and distinct one. Some of the variations observed are probably due to impure cultures, as isolation by the plating method cannot be relied upon always to yield thor-

oughly pure cultures. The characteristics of this group are essentially those of *B. cloacae*.

Kligler observes that gelatin liquefaction is correlated with a negative glycerol fermentation. Rogers, Clark and Evans, however, regarded 80 per cent of gelatin-liquefying lactose-fermenting strains isolated from grains, as glycerol fermenters. This difference, we think, is due to the employment of acid production in place of gas formation as an index of fermentation. Kligler selected 1.5 per cent, and Rogers 1 per cent normal acid as the line of demarcation between fermenters and non-fermenters. If the Kligler standard is applied to the Rogers' strains, only 2 or 3 (5.7–7.5 per cent) are glycerol fermenters, 3 or 4 (7.5–10 per cent) are questionable, while 34 (85.0 per cent) would be regarded as non-fermenters. Acid production as an index of fermentation is particularly unreliable with glycerol. The results obtained in this study, using gas formation as the criterion of fermentation, are well in accord with those of Kligler.

There were included in our series seven organisms which did not form gas from lactose. In all other characters they are like *B. cloacae*. Five of these strains gave a positive test for acetyl-methyl-carbinol from lactose. Of several thousand tests with coli-like organisms, these were the only instances (except one case of a starch reaction) in which a positive reaction for acetyl-methyl-carbinol was not accompanied by gas formation. It is of course possible that some gas was formed, but that the quantity was so small that it was entirely absorbed by the culture medium and therefore not apparent in the closed arm of the fermentation tube.

Differentiation of groups. Briefly stated, we may say that group A differs from B in that it is motile, and does not give the Voges-Proskauer reaction, nor form gas from corn starch. It differs from group C in that gelatin is not liquefied, the Voges-Proskauer reaction is negative, and gas is formed much more commonly from dulcitol and glycerol. The indol reaction is also usually positive for group A but negative for group C.

Group B differs from C in gelatin liquefaction, motility and

gas formation from many substances. Group B is non-motile and rarely liquefies gelatin. C is just the reverse. Gas is practically always formed from glycerol, dextrin and starch by the organisms in group B, but only rarely by those in group C.

Observations on acetyl-methyl-carbinol production from various substances

It was thought that acetyl-methyl-carbinol production from various substances (carbohydrates, alcohols and glucosides) might be an aid in the differentiation of the organisms.

Ferriera, Horta and Paredes indicate that *B. cloacae* differs from *B. aerogenes* by its ability to give the "Proskauer" reaction with galactose and mannitol. Their observations are not confirmed by this study.

Levine pointed out that coli-like forms which did not form acetyl-methyl-carbinol from glucose (Voges-Proskauer strains) rarely formed the carbinol from other fermentable substances, whereas the Voges-Proskauer-positive strains often gave a positive "carbinol" test with other carbohydrates, etc.

Of 45 Voges-Proskauer-negative cultures 2 gave a positive carbinol test with lactose and 1 with starch. With galactose, sucrose, raffinose, dulcitol, mannitol, glycerol, salicin, dextrin, and inulin the reaction was uniformly negative.

Among 139 Voges-Proskauer-positive strains, positive "carbinol" tests were obtained as follows: galactose 89 (64.0 per cent); lactose 83 (59.6 per cent); sucrose 124 (89.3 per cent); raffinose 114 (82.4 per cent); dulcitol 15 (10.8 per cent); mannitol 123 (88.6 per cent); glycerol 6 (4.3 per cent); salicin 94 (6.8 per cent); dextrin 14 (10.0 per cent); and starch 25 (18.0 per cent). Inulin was always negative. The positive reaction with glycerol is particularly interesting because it indicates a synthesis, the acetyl-methyl-carbinol containing one more carbon atom than glycerol.

In a general way, there is very little difference between the groups B and C with respect to formation of acetyl-methyl-carbinol from galactose, sucrose, raffinose, mannitol, and inulin.

Group B reacted positively in 79.6 per cent of the lactose and 56.8 per cent of the salicin tests, as compared with 48.8 per cent and 75.0 per cent respectively for group C. The latter never formed the carbinol from dulcitol and glycerol, and only 1.1 per cent of the strains were positive with dextrin and starch. With group B positive reactions were obtained as follows: dulcitol 34.1 per cent; glycerol 13.6 per cent; dextrin 29.6 per cent and starch 54.6 per cent. It should be noted that gas formation from dulcitol, glycerol and starch shows a much more striking differentiation between *B. cloacae* and *B. aerogenes* than does acetyl-methyl-carbinol production from these substances.

III. CONCLUSIONS

1. It appears that under the conditions prevailing in Ames, Iowa, coli-like bacteria were considerably more abundant in soils upon which crops were growing than in absolutely fallow areas receiving similar soil treatment.

2. The Voges-Proskauer and methyl-red reactions are well correlated.[1]

3. The *aerogenes-cloacae* types, which give a positive Voges-Proskauer reaction and are alkaline to methyl red in Clark and Lubs' peptone-di-potassium-phosphate-glucose solution, are the predominant coli-like forms in soil.

4. The *aerogenes-cloacae* group may be differentiated from the *coli* group by the methyl-red or Voges-Proskauer reaction. *B. aerogenes* differs from *B. cloacae* in that it is non-motile, rarely liquefies gelatin, and forms gas from glycerol and corn starch.

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[1] Since the completion of this paper Greenfield in Kansas, and Hulton in Pennsylvania have confirmed the correlation of the Voges-Proskauer and methyl-red reactions, as observed by Levine.

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THE EFFECT OF STERILIZATION UPON SUGARS IN CULTURE MEDIA

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A great many bacteria possess the power of fermenting carbohydrates. As a rule, a given species is able to ferment more than one kind of carbohydrate, but the various species are sufficiently limited and varied in this power so that they may often be distinguished and identified by studies of their fermentative abilities. To ascertain the fact whether a sugar has actually been fermented, it would, of course, be absolutely decisive to isolate, by chemical analysis, a product characteristic of the sugar in question. The problem, however, can not in general be solved in this way, since the work involved is very great. As a rule, it is sufficient to demonstrate the production of acid or of gas as a result of growth in the medium. Acid or gas production is not characteristic of any particular sugar. Especially in the case of disaccharides, gas and acid may be produced from the products of heat action on the disaccharide, whether or not they could be produced from the disaccharide intentionally introduced. It is therefore especially necessary, if one is to use the simpler test, to know how and to what extent the heat of sterilization acts on the sugars in question.

That disaccharides, on heating, hydrolize into their component sugars is a well-known fact. C. S. Butler (1913) states,

> "Some carbohydrates suffer inversion to a lower order if subjected to heat in the presence of weak acid or alkali, and in the finished product we find, for instance, that a supposed Shiga strain of the dysentery bacillus is producing acid in the maltose medium. As a matter of fact, it is simply showing its capacity to ferment glucose inverted from maltose."

It is the opinion among bacteriologists that maltose "breaks down" in this way even in the comparatively short period of sterilization. It was also thought that heating in the autoclave at fifteen pounds for fifteen minutes was more destructive to the sugars than heating in the Arnold for fifteen minutes on three successive days. So prevalent is this idea among bacteriologists that we now find in the text-books directions which say that "media which contain neither sugar nor gelatin . . . . may be sterilized in the autoclave at fifteen pounds for from fifteen minutes to one-half hour. Media which contain these must be sterilized by the fractional method" (Hiss and Zinsser, 1912). On the other hand, we are told that "in the breaking down of sugar media the time of sterilization has a greater effect than does the temperature" (Standard Methods of Water Analysis, 1912).

Since a great deal of dependence is placed on the fermentation of sugars, any knowledge concerning the chemical transformation of the sugars in the preparation of the media should be of value. It was the aim of this investigation: (a) to determine the behavior of sugars during sterilization; (b) to determine what form of sterilization is best suited to the sterilization of sugar media. Both chemical and bacteriological methods were employed to this end.

CHEMICAL METHODS

It was necessary to devise a method which would enable one to determine quantitatvely a disaccharide in the presence of a monosaccharide. The amount of monosaccharide would doubtless be very small, but it is a well-known fact that the presence of monosaccharides seriously interferes with the reactions of disaccharides. Attempts to remove one or more of the sugars by bacteria or yeasts, or to separate the sugars by means of the osazone reaction (Marquenne, 1891) met with no succcess.

Hinkel and Sherman (1907) have shown that a modification of the original Barfoed's solution can be used for the detection of monosaccharides. Their reagent is made as follows: 4.5 grams of copper acetate are dissolved in 100 cc. of water to

which have been added 2 cc. of a normal acetic acid solution. This solution must be stable enough to withstand heating for ten minutes on a boiling water bath. In the presence of monosaccharides the copper solution is reduced to cuprous oxide. This reddish precipitate is easily seen. Excessive boiling is apt to lead to hydrolysis of the disaccharide and care must be taken to avoid this. Each of the sugars to be studied gives, on hydrolysis, products which react with the above reagent.

Cohen used this method to distinguish between glucose and maltose in solutions containing less than 0.02 per cent of the former, and at least 0.2 per cent of the latter, "provided the tests are controlled by check experiments with known solutions of all the sugars." Roaf (1908) also studied the action of maltase on maltose by means of this solution, and was able to determine minute quantities of glucose in the presence of the maltose.

TECHNIQUE

Test tubes of large bore were used, since 25 to 30 cc. of liquid were to be tested. One per cent solutions of the various sugars in distilled water were prepared and sterilized in the autoclave for different periods of time, fifteen minutes, thirty minutes, one hour and two hours. Some of the solutions were also heated in the Arnold for fifteen, thirty, and forty-five minutes. Twenty cubic centimeters of the sugar solution to be tested were transferred to a test tube, and 5 cc. of the copper solution added. After a thorough shaking, the tubes were heated to 100°C. in a water bath for four minutes. A series of standards containing various amounts of the monosaccharides corresponding to the disaccharide used was treated in similar manner. If any reduction took place its extent could be estimated by comparing it with the standards. As the amount of reduction was sometimes very slight, the tubes were compared in a strong light against a black background.

The determinations were made on maltose, lactose, sucrose, and raffinose, since these are the polysaccharides most commonly used in bacteriological technique. All of these sugars were

Merck's Blue Label chemicals. The results are given in table 1. The figures represent the comparative degree of reduction. From this table it will be seen that of the four sugars, raffinose is the most stable; then follow sucrose, lactose, and maltose in the order named. Sucrose shows hydrolysis only after prolonged heating, but both lactose and maltose show hydrolysis in every case, with one possible exception. The action on lactose in the Arnold becomes evident only after the second heating. It will also be noticed that according to these figures the hydrolysis in the Arnold is greater than in the autoclave. This is a point of practical importance, and is contrary to most of the views

TABLE 1

Comparative amounts of monosaccharides detected by Barfoed's method

TIME	LACTOSE	MALTOSE	SUCROSE	RAFFINOSE
Autoclaved 15 minutes	Trace	1	0	0
Autoclaved 30 minutes	1.5	3	0	0
Autoclaved 1 hour	2	Very great	Slight	0
Arnold first day	0	4	0	0
Arnold second day	1	Very great	0	0
Arnold third day	2	Very great	0	0

held in regard to the two methods. As has been previously pointed out, the autoclave has long been considered as having the greater hydrolytic effect on the sugars, yet the time factor in these experiments is evidently more important than the temperature factor. These results obtained from a study of pure sugars in water solution would not necessarily apply to culture media. The chemical methods used are not applicable to culture media; and the behavior of the sugars in culture media was therefore studied by bacteriological methods.

BACTERIOLOGICAL METHODS

Stock solutions of nutrient broth were prepared as follows:

Liebig's extract	0.3 per cent
Witte's peptone	1.0 per cent
Sugars	1.0 per cent
Reaction	Neutral to phenolphthalein

The sugars were added to the broth just previous to tubing. They were then sterilized as follows: fifteen minutes, thirty minutes, one hour, and two hours in the autoclave; and fifteen minutes on three successive days in streaming steam in the Arnold sterilizer.

TABLE 2

Acid produced by fermentation in sugar media after sterilization. Acidity expressed in cubic centimeters N/20 NaOH in 5 cc. of medium

	CONTROL	TWENTY-FOUR HOURS		FORTY-EIGHT HOURS	
		Total acid	Difference*	Total acid	Difference
	cc.	cc.	cc.	cc.	cc.
Maltose inoculated with *B. dysenteriae*					
Autoclaved 15 minutes	0.38	0.96	0.58	1.55	1.17
Autoclaved 30 minutes	0.45	1.23	0.78	1.24	0.79
Autoclaved 1 hour	0.73	1.73	1.00	1.62	0.89
Autoclaved 2 hours	0.92	1.74	0.82	1.95	1.03
Arnold 3 days	0.35	1.23	0.88	1.71	1.36
Lactose inoculated with *B. paratyphosus*					
Autoclaved 15 minutes	0.40	0.55	0.15	0.46	0.06
Autoclaved 30 minutes	0.54	0.78	0.24	0.60	0.06
Autoclaved 1 hour	0.68	0.97	0.29	0.73	0.05
Autoclaved 2 hours	0.87	1.30	0.43	1.17	0.30
Arnold 3 days	0.41	0.60	0.19	0.46	0.05
Sucrose inoculated with *B. coli*					
Autoclaved 15 minutes	0.35	0.50	0.15	0.41	0.06
Autoclaved 30 minutes	0.37	0.46	0.09	0.46	0.09
Autoclaved 1 hour	0.39	0.55	0.16	0.47	0.08
Autoclaved 2 hours	0.40	0.56	0.16	0.52	0.12
Arnold 3 days	0.39	0.46	0.07	0.41	0.02

Acid is produced by the Shiga strain of *B. dysenetriae* from glucose, but not from maltose. Inoculations with this strain were employed in the maltose experiments as a test for the production of glucose by hydrolysis. Similarly *B. paratyphosus* was used to detect hydrolysis of lactose, and *B. coli* was used for sucrose.

* Difference is found by subtracting acid of control tube from total acidity.

After incubation for twenty-four hours, 5 cc. of the culture were titrated hot against N/20 NaOH, using phenolphthalein as an indicator. The results given in the tables are the averages of ten titrations, expressed in cubic centimeters. Control titrations were made on the uninoculated broth, and this acidity was eventually subtracted from the acidity determined at the end of the twenty-four and forty-eight hour periods. The difference between these two figures was taken as the acidity caused by the fermentation of the monosaccharide. Broth with no added sugar was also inoculated, to determine what influence the muscle sugar might have.

TABLE 3

Acid produced in nutrient broth inoculated with same organisms used in Experiment 2. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium*

	CONTROL	TWENTY-FOUR HOURS		FORTY-EIGHT HOURS	
		Total acid	Difference	Total acid	Difference
	cc.	cc.	cc.	cc.	cc.
Nutrient broth inoculated with *B. dysenteriae*					
Autoclaved 15 minutes	0.25	0.30	0.05	0.32	0.07
Autoclaved 30 minutes	0.21	0.35	0.14	0.36	0.15
Autoclaved 1 hour	0.26	0.34	0.08	0.34	0.08
Autoclaved 2 hours	0.22	0.36	0.14	0.36	0.14
Arnold 3 days	0.27	0.37	0.10	0.34	0.07
Nutrient broth inoculated with *B. paratyphosus*					
Autoclaved 15 minutes	0.25	0.42	0.17	0.37	0.12
Autoclaved 30 minutes	0.21	0.35	0.14	0.28	0.07
Autoclaved 1 hour	0.26	0.37	0.11	0.28	0.02
Autoclaved 2 hours	0.22	0.38	0.16	0.25	0.03
Arnold 3 days	0.27	0.36	0.09	0.27	0.00

Reference to the tables shows that sucrose gives no evidence of hydrolysis, whereas maltose and lactose show considerable hydrolysis, as evidenced by acid production. In the case of both of these sugars, more acid is produced in the tubes sterilized in the Arnold than in the tubes autoclaved for thirty minutes. Maltose seems to be broken down more than the lactose. This is in accord with the findings obtained by the chemical method applied to the pure sugar solutions.

An interesting fact was noticed in the case of lactose. The amount of acid found decreased during the second twenty-four hour period. The work of Kendall (1911) suggests the possibility that the organisms use up all the available sugar in the first twenty-four hour period, and next attack the protein,

TABLE 4

Acid produced by fermentation in sugar media after sterilization. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium*

	CONTROL	TWENTY-FOUR HOURS		FORTY-EIGHT HOURS	
		Total acid	Difference	Total acid	Difference
	cc.	cc.	cc.	cc.	cc.
Maltose inoculated with *B. dysenteriae*					
Autoclaved 15 minutes	0.34	0.53	0.19	0.86	0.52
Autoclaved 30 minutes	0.44	0.76	0.32	0.96	0.52
Autoclaved 1 hour	0.53	1.30	0.77	1.35	0.82
Arnold 3 days	0.40	0.45	0.05	1.18	0.78
Lactose inoculated with *B. dysenteriae*					
Autoclaved 15 minutes	0.14	0.26	0.12	0.25	0.11
Autoclaved 30 minutes	0.20	0.31	0.11	0.20	0.00
Autoclaved 1 hour	0.37	0.72	0.35	0.29	0.08
Arnold 3 days	0.22	0.33	0.11	0.76	0.54
Sucrose inoculated with *B. dysenteriae*					
Autoclaved 15 minutes	0.17	0.22	0.05	0.20	0.03
Autoclaved 30 minutes	0.13	0.24	0.11	0.30	0.17
Autoclaved 1 hour	0.14	0.21	0.07	0.28	0.14
Arnold 3 days	0.12	0.20	0.08	0.33	0.21
Raffinose inoculated with *B. coli*					
Autoclaved 15 minutes	0.24	0.40	0.16	0.22	0.02
Autoclaved 30 minutes	0.26	0.40	0.14	0.35	0.09
Autoclaved 1 hour	0.29	0.36	0.07	0.36	0.07
Arnold 3 days	0.29	0.31	0.02	0.27	−0.02

setting free ammonia, which neutralizes the acid originally produced.

The amount of acid produced in maltose solutions is considerable, and it was thought that the stock sample might be somewhat hydrolyzed. A fresh supply was obtained from Merck and the tests were conducted as follows: Maltose, lactose, and

sucrose were inoculated with the "Y" Hiss strain of *B. dysenteriae;* the raffinose was inoculated with *B. coli.* The methods used were the same as those of the previous experiments. The results are found in tables 4 and 5, and it will be seen that they are similar to those of the first experiment.

The sterilization of sugar media by heating methods is evidently very destructive to the disaccharides, maltose and lactose. Media containing these sugars can be sterilized by filtering through unglazed porcelain. It might be expected that little or no chemical change would be produced by this treatment.

TABLE 5

Acid produced in nutrient broth inoculated with same organisms used in Experiment 4. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium.*

	CONTROL	TWENTY-FOUR HOURS		FORTY-EIGHT HOURS	
		Total acid	Difference	Total acid	Difference
Nutrient broth inoculated with *B. dysenteriae*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Autoclaved 15 minutes	0.03	0.20	0.17	0.27	0.24
Autoclaved 30 minutes	0.10	0.18	0.08	0.29	0.19
Autoclaved 1 hour	0.11	0.24	0.13	0.28	0.17
Arnold 3 days	0.10	0.26	0.16	0.29	0.19
Nutrient broth inoculated with *B. coli*					
Autoclaved 15 minutes	0.03	0.31	0.28	0.22	0.19
Autoclaved 30 minutes	0.10	0.33	0.23	0.21	0.11
Autoclaved 1 hour	0.11	0.35	0.24	0.23	0.12
Arnold 3 days	0.10	0.40	0.30	0.24	0.14

Accordingly, an experiment was conducted in which two lots of the same medium containing lactose were sterilized by two different methods. One part was heated for fifteen minutes at 15 pounds pressure in the autoclave. The other part was sterilized by filtering through a Chamberland filter. Every precaution was taken to keep the medium sterile. The tubes were placed in the incubator for twenty-four hours, and those showing contamination were discarded. These two lots of media were then inoculated with *B. paratyphosus B.*

TABLE 6

Comparison of autoclaving and filtering. Acidity expressed in cubic centimeters N/20 NaOH in 5 cc. of medium

	CONTROL TUBE	INOCULATED TUBE	DIFFERENCE
	cc.	cc.	cc.
Filtered broth	0.10	0.20	0.10
Heated broth	0.45	0.92	0.47

The filtered broth showed an acid production of 0.1 cc. N/20 which is about the limit of titration, while the broth heated in the autoclave titrated 0.4 cc. of alkali. It is evident that filtering the medium is a more sparing procedure.

THE INFLUENCE OF STERILIZATION ON THE REACTION OF SUGAR MEDIA

In the foregoing experiments the medium before sterilization was "neutral" (faintest tinge of pink to phenolphthalein). During sterilization the media which contained sugar became acid, the acidity increasing with the time. This had not been the case with peptone broth containing no sugar. The sugar solution in distilled water used for the Barfoed's test produced no acid during two hours sterilization in the autoclave. It thus appeared that both sugar and peptone, or other active substance in the peptone broth, are necessary for the development of acidity during sterilization.

A table (table 7) was compiled from data presented above. It will be noticed that peptone gives more acidity than the beef extract; the nutrient broth about the same as the peptone. However, when a sugar is added a great difference is noted. The addition of maltose or lactose gives rise to considerable acidity on sterilization. Sucrose shows some acidity and raffinose but little. It is interesting to note that the sugars which give the greatest acidity on sterilization are the ones which have been found above to break down most readily.

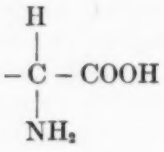
Hugo Schiff (1901–1902) showed that there is a reaction between formaldehyde and the amino group. In the amino acids, which

TABLE 7

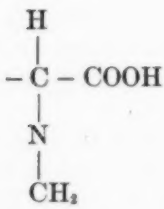
Acid produced in sterilizing various media. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium*

	MEAT EXTRACT	PEPTONE	NUTRIENT BROTH	MALTOSE BROTH	LACTOSE BROTH	SUCROSE BROTH	RAFFINOSE BROTH
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Autoclaved 15 minutes	0.13	0.24	0.25	0.38	0.40	0.35	0.25
Autoclaved 30 minutes	0.13	0.30	0.21	0.45	0.54	0.37	0.23
Autoclaved 1 hour	0.13	0.26	0.26	0.73	0.68	0.39	0.28
Autoclaved 2 hours	0.16	0.29	0.22	0.92	0.87	0.40	0.32
Arnold 3 days	0.13	0.28	0.27	0.35	0.41	0.39	0.32

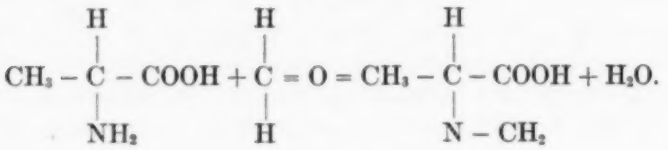
contain the carboxyl group (COOH) and the amino group (NH_2) Schiff was able to neutralize the influence of the amino part on the carboxyl group by treating with neutral formaldehyde. The grouping



is changed to



Thus alanine, when treated with formaldehyde, is converted into methylene-alanine and water as follows:



Neutral solutions of amino acids are rendered acid on the addition of neutral formaldehyde. Since sugar may be considered as a polymer of formaldehyde and has similar reacting groups, it might easily be imagined that it would follow reactions similar to those mentioned above.

An experiment was devised to test this point. A 0.5 per cent solution of alanine was prepared, together with a 1 per cent solution of some old maltose, new maltose and raffinose. Solutions were then made which contained 0.5 per cent of alanine and 1 per cent of the sugars mentioned. Portions of each of these solutions were sterilized in the autoclave for periods of fifteen minutes, thirty minutes, and one hour. Five titrations were made on each solution, and the results are given in table 8. The titrations were made as in the previous experiments.

TABLE 8

The effect of alanine on the production of acid from sugars by autoclaving. Acidity expressed as cubic centimeters N/20 NaOH *per 5 cc. solution*

	LENGTH OF STERILIZATION		
	Fifteen minutes	Thirty minutes	One hour
	cc.	cc.	cc.
Alanine	0.00	0.00	0.00
Old Maltose	0.00	0.00	0.00
Alanine + Old Maltose	0.00	0.34	0.46
Fresh Maltose	0.00	0.00	0.00
Alanine + Fresh Maltose	0.00	0.10	0.30
Raffinose	0.00	0.00	0.00
Alanine + Raffinose	0.00	0.00	0.00

All of the solutions were neutral to phenolphthalein at the start of the experiment. The results show that the alanine alone, and the sugar alone, give no acidity. But in those solutions containing the mixture of alanine and maltose, acid is produced. The mixture of alanine and raffinose is neutral, even after sterilization for an hour. The production of acid is not the only index of chemical change. Those solutions which become acid also become colored. The depths of color, as well as the acid, increased as the length of sterilization was increased.

A similar experiment was performed, using maltose, lactose, and raffinose in solution with asparagin. The results were like those of the previous experiment.

TABLE 9

The effect of asparagine on the production of acid from sugars by autoclaving. Acidity expressed as cubic centimeters N/20 NaOH *per 5 cc. solution*

	LENGTH OF STERILIZATION		
	Fifteen minutes	Thirty minutes	One hour
	cc.	*cc.*	*cc.*
Asparagine	0.00	0.00	0.00
Maltose	0.00	0.00	0.00
Asparagine + Maltose	0.00	0.44	0.58
Lactose	0.00	0.00	0.00
Asparagine + Lactose	0.00	0.34	0.64
Raffinose	0.00	0.00	0.00
Asparagine + Raffinose	0.00	0.00	0.00

These experiments seem to bear out the fact previously indicated—that it is the presence of an unstable sugar molecule together with an amino acid which gives rise to the acidity of sterilization.

SUMMARY AND CONCLUSIONS

An attempt has been made to determine the extent of hydrolysis of certain disaccharides commonly used in bacteriological investigations, with the following results:

By means of Barfoed's method maltose and lactose were found to be hydrolyzed to a considerable extent in water solution. Sucrose and raffinose did not break down.

Heating in streaming steam for three successive days in the Arnold seems to hydrolyze lactose and maltose more than heating in the autoclave at 15 pounds for fifteen minutes.

By the use of bacterial cultures the hydrolysis of these sugars was studied in culture media, with similar results.

Peptone broths containing the sugars mentioned were subjected to sterilization in the Arnold in streaming steam and to various heatings in the autoclave at 15 pounds pressure. They

were then inoculated with strains of bacteria which would not ferment the sugar introduced.

Lactose and maltose broth, so inoculated, were found to be fermented by the organisms introduced. The amount of fermentation increased as the sterilization increased. It is assumed that this fermentation is due to the presence of monosaccharides in the broth.

By filtering sugar media this hydrolysis of the sugar can be avoided.

The sugars found above to be easily hydrolizable, namely, maltose, and lactose, formed acid when heated in culture media, or with alanine or asparagin in water solution; whereas raffinose, which is resistant to hydrolysis, formed no acid under like circumstances. The acidity produced in sterilization seems to be due to a reaction between the sugar and the amino groups.

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THE PRESENCE OF THE B. COLI AND B. WELCHII GROUPS IN THE INTESTINAL TRACT OF FISH (STENOMUS CHRYSOPS)

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The *B. coli* group, the *B. Welchii* group and other lactose-fermenting organisms have long been regarded as normal inhabitants of the intestinal tract of the homoiothermal or warm-blooded animals, and their presence outside of the body has been regarded as a very strong index of the presence of fecal pollution from those animals. The presence of these microorganisms in the intestinal tract of the poecilothermal or cold-blooded animals, however, has been a subject for dispute. A review of the literature does not seem to help the situation in the least, for we find the authorities evenly divided on the subject. The tendency in England and America seems to be to regard the *B. coli* group as the normal inhabitants of the higher animals only, while on the continent of Europe, especially in Germany, the interpretation has not been so strict. No opinions concerning the status of the *B. Welchii* group could be found, and such would be difficult to form because of the spore-forming abilities and anaerobic conditions necessary for the growth of that group of organisms.

All workers seem to agree, however, that the *B. coli* group is much more constant in the intestinal tract of the higher animals than of the cold-blooded animals. Hoag (1899) was able to isolate members of the *B. coli* group from the fish which he examined. Amyot (1902) examined the intestinal contents of 23 fishes, representing 14 species taken from Lake Erie, and was unable to isolate any lactose fermenters of the colon type.

Houston (1903) found members of the colon group in 13 per cent of the fishes examined, but concludes that the *B. coli* group is "either absent or present in small numbers in the intestines of fishes dredged from the sea in localities remote from sewage pollution." Whipple (1904) was unable to find the colon group in the intestinal tract of the trout, perch, and sunfish, taken from unpolluted waters, while Johnson (1904) was able to isolate these organisms 47 times out of the 67 fishes examined from polluted waters. Eyre (1904) isolated the members of the *B. coli* group from the intestinal tract of the sprat, dab, smelt, mullet, sole, skate and dogfish taken from traps two miles from the shore in unpolluted waters. Van Mallannah in an unpublished work reports positive results in 37.5 per cent of 32 fish examined, while Fromme states that 41 per cent of fishes examined responded positively to the *B. coli* tests. He concludes, however, that the *B. coli* group is only rarely present in the intestinal tract of the cold-blooded animals, while they are much more constant in the higher vertebrates. Bettencourt and Borges (1908) isolated 29 cultures of colon-like bacilli from the intestinal tract of 17 fishes, reptiles and amphibia, of which only two proved to be members of the colon group, although in these tests no enrichment medium was employed.

The purpose of this paper is to present some results as to the presence of the *B. coli* group and the *B. Welchii* group in the intestinal contents of fishes which were obtained at the Woods Hole Laboratory of the Bureau of Fisheries during the summer of 1916, while the writer was making an intensive study of the Bacteriology of the Food Fishes, especially the scup (*Stenomus Chrysops*).

The scup belong to the family Sparidae and to that large group of fishes known as "bottom feeders" such as the porgies, sea robins, drum fishes, tautog and sea bass which obtain their food supply from the floor of the ocean; hence, the character of the bottom on which the fish feed will influence the bacterial flora of their intestinal tract. Peck (1895) found that the food of the scup was somewhat varied but generally characteristic of the bottom fauna and flora. He found "at times very many

sand dollars (*Echinarachnius parma*) ground up with the sand and deep black mud of the bottom from which they were feeding, just above which the amphipods are usually abundant." Young specimens have often been found near the shores of coves and harbors feeding on young fishes.

The scup used in the following experiments were taken from the waters in and around the Woods Hole Laboratory of the Bureau of Fisheries. In some cases the fishes examined came from the government traps situated in Buzzards Bay in waters which were always coli-negative, and in other cases they came from the traps of the Marine Biological Laboratory situated either in Buzzards Bay or Martha's Vineyard Sound, both of which were free from any bacteriological evidence of fecal pollution, while other fish were caught by hook and line in Laky's Bay, an arm of Martha's Vineyard Sound, south of the Marine Biological Laboratory traps. The depth of the water from which the fishes were taken varied from a few feet near the shore in case of the traps to over 40 feet in Laky's Bay. In general the bottom seemed to be rocky and the waters were always free from the presence of lactose fermenters of intestinal origin (as shown by formation of gas in lactose broth).

The fishes were brought to the laboratory in clean baskets just as soon as possible after they were caught, and the following examination was made. After the scales were removed, the skin was thoroughly washed with 95 per cent alcohol and ignited. An incision was then made through the skin into the peritoneal cavity and the intestinal tract carefully dissected out, using instruments which were sterilized by constantly dipping in 95 per cent alcohol and lighting in the flame. As soon as the intestinal tract was dissected out, its exterior was seared with a hot scalpel at a distance of about two inches from the anus and then an incision was made with sterile scissors and the intestinal content allowed to drop into tubes of lactose broth, which were incubated at 37°C. It was impossible to graduate exactly the amount of fecal material which dropped into the tubes, but generally 5 to 10 grams of the intestinal tract content was used. Tubes which showed a posi-

TABLE

	LOT NUMBER												TOTAL
	1	2	3	4	5	6	7	8	9	10	11	12	
Source	B. T.	A.	S. T.	S. T.	B. T.	B. T.	B. T.	B. T.	B. T.	V. S.	V. S.	V. S.	
Date	7/20	7/25	7/27	8/2	8/5	8/7	8/21	8/22	8/23	8/24	8/29	8/30	
Number of fish examined	10	7	2	8	8	6	5	6	5	8	21	7	93
Gas in lactose broth:													
Positive	4	6	2	6	7	4	4	4	4	7	19	6	73(78.5%)
Negative	6	1	0	2	1	2	1	2	1	1	2	1	20(21.5%)
B. coli group isolated	4	6	1	2	4	0	2	2	2	4	7	3	37(39.8%)
Alone	4	5	1	2	4	0	2	2	2	1	1	3	27(29.0%)
With *B. Welchii*	*	1	0	0	0	0	0	0	0	3	6	0	10(10.8%)
B. Welchii group isolated	*	1	0	1	2	1	0	1	0	4	16	2	28(30.1%)
Alone	*	0	0	1	2	1	0	1	0	1	10	2	18(19.3%)
With *B. coli*	*	1	0	0	0	0	0	0	0	3	6	0	10(10.8%)
Condition of water	0	+	0	0	0	0	0	0	0	0	0	0	
Total positive fish (Welchii or Coli)	4	6	1	3	6	1	2	3	2	5	17	5	55(59.2%)
Total negative fish	6	1	1	5	2	5	3	3	3	3	4	2	38(40.8%)

* No examination.

S. T. = Sound trap; A. = Aquarium; B. T. = Bay trap; V. S. = Vineyard Sound; + = *B. coli* present.

tive gas production by the forty-eighth hour were plated out on litmus lactose agar, and transplants were identified as members of the *B. coli* group. A Gram-negative, rounded and aerobic bacillus, producing gas and acid in lactose broth, and no liquefaction of gelatine, was taken as the criterion of membership in the *B. coli* group. All tubes which showed gas were examined for the *B. Welchii* group by the following procedure. One cubic centimeter of the lactose broth tube showing gas production was inoculated into a tube of litmus milk which was then sealed with paraffin. These tubes were heated at a temperature of 80°C. for ten minutes after which they were incubated at 37°C. for forty-eight hours. Vigorous gas production, with the subsequent expulsion of the paraffin plug, acid reaction, "stormy fermentation" and odor of butyric acid were regarded as indicative of the presence of the *B. Welchii* group.

The results obtained by the above bacteriological examination of the intestinal content of the scup for the presence of the *B. coli* group and the *B. Welchii* group may be summarized in the accompanying table.

CONCLUSIONS

The intestinal content of 93 scup (*Stenomus chrysops*) taken from unpolluted waters in and about the Woods Hole Laboratory of the Bureau of Fisheries was examined bacteriologically for the presence of the *B. coli* and the *B. Welchii* group with the following results:

1. The intestinal content of 73 (78.5 per cent) scup examined contained lactose-fermenting organisms.

2. Members of the *B. coli* group were isolated from the intestinal content of 37 (39.8 per cent) of the scup examined, in 27 (29.0 per cent) cases alone and in 10 (10.8 per cent) cases in company with members of the *B. Welchii* group.

3. The members of the *B. Welchii* group were isolated from the intestinal content of 28 (30.1 per cent) of the scup examined, in 18 (19.3 per cent) cases alone and in 10 (10.8 per cent) cases in company with the members of the *B. coli* group.

4. The intestinal content of 55 (59.2 per cent) of the scup examined contained organisms of either the colon or the Welchii type.

5. The presence of bacteria in the intestinal tract of the scup seems to be correlated with the amount and type of food present.

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NITROGEN-ASSIMILATING ORGANISMS IN MANURE[1]

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In a recent article from the Wisconsin Agricultural Experiment Station, Tottingham[2] called attention to a gain in nitrogen in fermenting manures. Because of the practical importance of stable manure for agriculture, it was thought advisable to study the nature of the organisms concerned with the fixation of nitrogen in manures.

EXPERIMENTS AND RESULTS

During the winter of 1916 experiments were planned to determine the occurrence and nitrogen-assimilating power of the organisms in manure. Samples of manure were prepared as follows: A mixture of manure consisting of two parts of fresh horse manure and one part of fresh cow manure was added to finely chopped wheat straw. One-half kilo of straw was taken to every 6.5 kilos of the horse-cow manure mixture.

The presence of nitrogen-fixing organisms was determined by the gain in nitrogen in liquid and solid cultures. One hundred cubic centimeter portions of mannitol solution, in one liter Erlenmeyer flasks, were inoculated with 10 grams each of the manure mixture. Immediately after inoculation one-half of the cultures were treated with strong sulphuric acid or sterilized in the autoclave. These served as controls. The remaining cultures were incubated for varying intervals of time. At the end of the

[1] Published by permission of the Director of the Wisconsin Agricultural Experiment Station.

[2] TOTTINGHAM, W. E. 1916 Jour. Biol. Chem., v, 24, no. 3, p. 221.

incubation period determinations of total nitrogen content were made on the controls, as well as on the cultures. The difference in the amount of nitrogen shows the increase due to biological factors.

EFFECT OF AGE OF MANURE ON NITROGEN FIXATION

The manure mixture was prepared as previously described, stored at room temperature, and at intervals of two and eight weeks composite samples were weighed into the mannitol solution. In each test 10 flasks were used, 5 controls and 5 cultures. The controls received 25 cc. of concentrated sulphuric acid immediately after inoculation. The cultures were kept at 28°C. At the end of three weeks, total nitrogen analyses were made of the entire contents of the flask. Table 1 shows the milligrams of nitrogen in each culture inoculated with manure two and eight weeks old. In mannitol solution manure two weeks old gave an increase of 6 mgm.; eight weeks old an increase of 6.4 mgm. of nitrogen. The age of the manure, at least within a certain range, apparently has very little effect on the gain in nitrogen. In the manure itself Tottingham found that after

TABLE 1

Effect of age of manure on gain in nitrogen

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION					
		Two weeks old			Eight weeks old		
			Average	Gain		Average	Gain
		mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
1	Control	37.5			68.7		
2	Control	37.9			68.0		
3	Control	37.1	37.4		64.7	66.8	
4	Control	37.2			65.9		
5	Control	37.1					
6	Bacteria	43.1			73.1		
7	Bacteria	43.6			73.0		
8	Bacteria	43.6	43.4	6.0	73.1	73.2	6.4
9	Bacteria	43.3			73.8		
10	Bacteria	44.2					

two to four weeks of fermentation there was a gain in the absolute amount of nitrogen; after twelve weeks, a loss. The increase in nitrogen due to the organisms of fermenting manure compares favorably with that obtained with the organisms of field soil. For example, 10 grams of soil in 100 cc. of mannitol solution gave an increase in nitrogen of 6 to 10 mgm.[3] The apparent increase in the nitrogen of the older sample of manure is no doubt due to a difference in the moisture content. At the time of inoculation the fresh manure mixture contained much more water than the old mixture.

EFFECT OF TEMPERATURE OF INCUBATION ON GAIN IN NITROGEN

In order to determine the temperature suitable for the growth of the nitrogen-assimilating organism or organisms of manure, when inoculated in mannitol solution, duplicate sets of cultures were prepared. One set was kept at 28°C. and the other was incubated at 37°C. The manure inoculum was eight weeks old. Nitrogen analyses after twenty-one days gave the results indicated in table 2. The data show that the organisms of manure fix more nitrogen at 28°C. than at 37°C.

TABLE 2

Effect of different temperatures of incubation on gain in nitrogen

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION					
		Incubated at 28°C.			Incubated at 37°C.		
			Average	Gain		Average	Gain
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	Control	41.9			41.9		
2	Control	42.0	41.9		42.0	41.9	
3	Control	41.7			41.7		
4	Bacteria	51.4			45.6		
5	Bacteria	48.1	49.7	7.8	47.5	46.5	4.6
6	Bacteria	49.7			46.5		

[3] Unpublished results of this station.

EFFECT OF HEAT ON THE NITROGEN-ASSIMILATING ORGANISMS OF MANURE

It was next arranged to study the relation of the nitrogen-fixing organisms of manure to partial sterilization. Six flasks of mannitol solution were inoculated with the manure mixture and then heated to 75°C. for ten minutes. If the gain in nitrogen is due to a spore-bearing organism, it is probable that this treatment will not have any decided effect on the amount of nitrogen assimilated. The data of table 3 furnish an answer to the question. The total gain after eighteen days' incubation at 28°C. amounted to 2.2 mgm. or about one-third the amount fixed in the unheated flasks. Apparently fermenting manure contains a nitrogen-fixing organism which is resistant to heat—a spore-forming organism. However, the total gain in nitrogen from the resistant organism, or organisms, is far less than that from the natural flora. The evidence indicates the presence of several kinds of nitrogen-fixing organisms.

TABLE 3

Effect of heating manure on the nitrogen-fixing flora

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION		
		Heated to 75°C.		
			Average	Gain
		mgm.	mgm.	mgm.
1	Control	41.9		
2	Control	42.0	41.9	
3	Control	41.7		
4	Bacteria	43.8		
5	Bacteria	43.9	44.1	2.2
6	Bacteria	44.5		

EFFECT OF TIME OF INCUBATION ON NITROGEN FIXATION

Microscopic examinations of the mannitol solution inoculated with manure showed a profuse growth of microorganisms, largely bacilli. About fifteen days after inoculation the bacterial film became covered with a mold growth. An attempt was

made to measure the relation of the molds to the increase in nitrogen. Advantage was taken of the slow development of the molds in comparison with the bacteria. Cultures were prepared and incubated for nine and twenty-one days.

Nitrogen assimilation takes place independently of mold growth, as shown in the figures of table 4. The total number of milligrams fixed was almost as great after nine days as after fifteen days.

Attention was next directed to the gain in nitrogen with pure cultures isolated from the manure mixture.

TABLE 4

Effect of time of incubation on the nitrogen-fixing flora

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION					
		Incubated for nine days			Incubated for twenty-one days		
			Average	Gain		Average	Gain
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	Control	35.3			37.5		
2	Control	36.7			37.9		
3	Control	36.6	36.3		37.1	37.4	
4	Control	36.1			37.2		
5	Control	36.6			37.1		
6	Bacteria	40.6			43.1		
7	Bacteria	41.0			43.6		
8	Bacteria	42.3	41.5	5.2	43.6	43.4	6.0
9	Bacteria	42.2			43.3		
10	Bacteria	41.3			44.2		

NITROGEN FIXATION WITH PURE CULTURES OF BACTERIA ISOLATED FROM MANURE

Here again, inoculations from the manure were made into mannitol solution. The cultures were examined microscopically at various intervals. During the early stages of growth gas bubbles appeared on the top of the liquid, and later there developed a tough, gelatinous film on the surface. At first the film consisted solely of bacteria, later of bacteria and molds. Microscopic mounts made from the film and treated with Gram's

iodine solution showed the presence of organisms which stained a faint golden color; but Azotobacter organisms were never found.

Dilutions were made using mannitol agar and mannitol-manure-extract agar. The last medium was made by replacing the distilled water with a water extract from the manure. A series of plates was poured. From these plates isolated colonies were transferred to agar slopes. In every case there developed a more abundant growth on the mannitol-manure agar than on the mannitol agar alone. As soon as the agar slopes showed abundant growth, a loopful of the culture was shaken vigorously with sterile water and sand and then replated.

Because of their frequent occurrence in plate cultures, four organisms were selected for the first test. These were designated as numbers 1, 2, 3, and 4.

The nitrogen-assimilating power of cultures numbers 1, 2, 3, and 4 was tested on mannitol films. For this purpose 100 cc. portions of mannitol agar in one liter Erlenmeyer flasks were used. The flasks were inoculated with a 1 cc. suspension of the various organisms. The cultures were incubated at 28°C. and at varying intervals enough sterile water added to keep the surface moist. Nitrogen assimilation was studied on mannitol-manure-extract agar in addition to mannitol agar. Microscopic examination showed moderate growth on the mannitol agar and profuse growth on the mannitol-manure extract agar. After twenty-one days of incubation the cultures were analyzed for total nitrogen. All of the organisms assimilated a small amount of nitrogen. On mannitol agar the gain amounted to about 1.5 mgm.; on mannitol-manure extract agar about 2 mgm.

In view of the slight gain in nitrogen of cultures 1, 2, 3, and 4, it was decided to attempt to isolate other forms from the manure mixture.

Three organisms, 5, 6, and 7 were isolated and their nitrogen-assimilating power studied. The average of five parallel analyses showed that culture 5 fixed 4.7 mgm. of nitrogen on mannitol-manure-extract agar and only 1.7 mgm. on mannitol agar.

TABLE 5

Nitrogen assimilation by culture 5 on mannitol-manure-extract agar

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF AGAR		
		Culture 5		Gain
			Average	
		mgm.	*mgm.*	*mgm.*
1	Control	16.5		
2	Control	16.5		
3	Control	16.3	16.5	
4	Control	16.3		
5	Control	16.9		
6	Bacteria	21.5		
7	Bacteria	21.0		
8	Bacteria	21.3	21.2	4.7
9	Bacteria	21.2		
10	Bacteria	21.0		

A repetition of this test on agar films and liquid cultures of mannitol-manure-extract gave similar results. Organism 5 when grown in mannitol-manure-extract medium, liquid or solid, assimilated about 4.5 mgm. of nitrogen. Although the gain in nitrogen is not as great, this organism compares very favorably with Azotobacter. It is no doubt one of the chief factors in the assimilation of nitrogen in manure.

The same procedure was followed in studying nitrogen fixation with cultures 6 and 7. Culture 7 failed to show any in-

TABLE 6

Nitrogen assimilation by culture 6 on agar and in solution

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION					
		Mannitol-manure extract agar			Mannitol-manure extract solution		
			Average	Gain		Average	Gain
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	Control	13.4	13.3		13.5	13.5	
2	Control	13.2			13.5		
3	Bacteria	18.5	19.0	5.7	16.7	16.6	3.1
4	Bacteria	19.5			16.5		

crease in nitrogen. The increase in nitrogen due to the growth of culture 6 is clearly seen from the figures in table 6. When grown on agar films the organism fixed more nitrogen than in solution.

A review of the data obtained with pure cultures shows that the gain in nitrogen is not as great as that obtained with manure itself. Apparently the presence of solid manure in the solution is not essential to nitrogen fixation. This fact is brought out very clearly by results presented in table 7. Here a water suspension of the manure was used to inoculate sterile mannitol-manure-extract. After sixteen days the increase in nitrogen amounted to 8.2 mgm.

TABLE 7

Nitrogen assimilation in mannitol-manure extract solution inoculated with manure

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION		
			Average	Gain
		mgm.	*mgm.*	*mgm.*
1	Control	9.5		
2	Control	9.5		
3	Control	9.4	9.3	
4	Control	9.1		
5	Control	8.9		
6	Bacteria	17.4		
7	Bacteria	17.6		
8	Bacteria		17.5	8.2
9	Bacteria	17.3		
10	Bacteria	17.7		

COMPARISON OF THE AMOUNT OF NITROGEN ASSIMILATED BY AZOTOBACTER AND BY ORGANISMS FROM MANURE

In this test a pure culture of Azotobacter isolated from garden soil was used to inoculate flasks of mannitol-manure agar. The same plan was followed as in previous experiments. Three weeks after inoculation, the Azotobacter cultures were analyzed. Table 8 gives the results of the total nitrogen determinations. Azotobacter fixed 8 mgm. of nitrogen. A comparison of the gain in nitrogen with manure bacteria and with Azotobacter fails to show any marked difference.

TABLE 8

Nitrogen assimilation by Azotobacter in mannitol-manure extract agar

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF AGAR		
		Azotobacter culture		Gain
			Average	
		mgm.	*mgm.*	*mgm.*
1	Control	12.6		
2	Control	12.7		
3	Control	12.5	12.6	
4	Control	12.6		
5	Control	12.6		
6	Bacteria	20.3		
7	Bacteria	20.6		
8	Bacteria	20.3	20.6	8.0
9	Bacteria	20.7		
10	Bacteria	20.9		

NITROGEN ASSIMILATION BY A MIXTURE OF THE PURE CULTURES ISOLATED FROM MANURE

Since the tests with pure cultures of organisms from manure failed to show a large gain in nitrogen, another experiment was carried out to study the nitrogen-fixing power of various combinations of organisms 5, 6, and 7. The results of this study are shown in table 9.

TABLE 9

Nitrogen assimilation by a mixture of pure cultures

TREATMENT	NITROGEN IN 100 CC. OF SOLUTION						
						Average	Gain
	mgm.					*mgm.*	*mgm.*
Control	9.5	9.5	9.4	9.1	8.9	9.3	
Culture 5	12.3	12.5	12.3	12.6	12.5	12.4	3.1
Culture 6	11.7	11.6	11.9	11.5	11.4	11.6	2.3
Culture 7	9.8	9.4	9.6	9.3	8.9	9.4	0.1
Cultures 5 and 6	12.4	12.3	12.8	13.1		12.7	3.4
Cultures 5 and 7	12.3	11.9	12.5	12.0	12.4	12.2	2.9
Cultures 6 and 7	12.0	12.0	11.8	11.7	12.0	11.9	2.6
Cultures 5, 6, and 7	11.8	11.6	11.8	11.7	11.8	11.7	2.4

From the data it is very evident that organism 5 in pure culture fixed as much or more nitrogen than in mixed cultures containing organisms 6 and 7. Apparently there are present in fermenting straw manure several nitrogen-fixing organisms. Among these 5 plays an important part.

CLASSIFICATION

Since culture 7 failed to show any decided gain in nitrogen, it was not included in this study. Cultures 5 and 6 apparently belong to the same group. Therefore, data are presented for only one culture.

From a review of the literature, it appears that the organism responsible for a large part of the gain in nitrogen in manure is an undescribed form. The specific name, *azophile*, is suggested. In relation to nitrogen fixation *B. azophile* is of especial interest. In fermenting manure or in culture media this organism will assimilate large amounts of atmospheric nitrogen. The total gain in nitrogen with pure cultures of *B. azophile* is almost as large as that obtained with Azotobacter.

Although data concerning the general occurrence of this organism are not ready for publication, it seems very probable that *B. azophile* is widely distributed in nature. The subject of nitrogen-fixing bacteria of manure, is receiving further study.

MORPHOLOGY AND PHYSIOLOGY OF BACTERIUM AZOPHILE, N. SP.

Morphological characters

The organisms are rods, occur singly with rounded ends, 0.7 to 0.8 micron wide by 1.4 to 1.8 microns long. Capsules are formed when the organism is grown on mannitol-manure-extract agar. No spores have been observed. It is motile in the hanging drop, is Gram-positive, and stains easily with ordinary aniline dyes.

Cultural characters

Agar plates. On plain agar at 28°C. surface colonies are less than 1 mm. in diameter in twenty-four hours. After three days the diameter increases, in some cases to 3 mm. Colonies are round, smooth, convex, and glistening. On mannitol-manure-extract agar growth develops much faster and is more profuse. In five days the growth with the extract agar is very profuse. When a loop is inserted growth does not appear viscous but can be picked up in cakes. On mannitol agar the growth is slower than on plain agar, finally attaining a rather heavy growth after five to six days.

Gelatin stab. Gelatin is liquefied completely, the liquefaction being of medium rapidity. Growth downward is very slow. It develops on surface.

Agar stabs. Growth is 2 mm. or more at surface in twenty-four hours. It remains at surface, is smooth and shining.

Agar slants. On bouillon agar a light orange pigment develops. Growth is abundant and wrinkled. On mannitol agar and mannitol-manure-extract agar growth is smooth. With the latter medium the growth is very profuse.

Mannitol-manure-extract solution. In mannitol-manure-extract solution, after twenty-eight days, growth appears very heavy and liquid begins to "lopper," provided the solution is maintained in shallow layers.

Beef bouillon. A heavy membranous growth is developed which does not fall to the bottom readily.

Potato slopes. Growth is moderate and does not spread over entire surface. It is elevated, shining, and of a brownish color. The slopes are not softened in ten days. A diastatic action is shown when tested with Fehling's solution.

Milk. Milk begins to digest in four to six days. A gelatinous pellicle is formed near the surface. Digestion continues downward slowly. Milk does not seem to curdle. No acid is produced in litmus milk.

Fermentation tubes. No gas is formed with sucrose, glucose, or lactose in bouillon. Growth takes place in all three media, but only on the surface. No acid is produced.

Nitrate broth. Nitrates are reduced to nitrites and finally to gaseous nitrogen or oxides of nitrogen.

Peptone solution. Heavy growth is developed near surface which later falls to bottom. Indol is produced.

Glycerol solution. Heavy membranous growth results. No acid is found when tested with blue litmus paper.

Aerobism. The organism is apparently a strict aerobe. It grows only in the open end of fermentation tubes, with ordinary sugars present; in agar stab, growth is on the surface only, and not at junction made by pouring melted agar on a column of solid agar. This organism's group number is 211.3331[4]23 according to the chart of the Society of American Bacteriologists.

SUMMARY

1. Nitrogen assimilation in fermenting manures is due to the activities of certain forms of bacteria.

2. Apparently there are several groups of bacteria concerned with nitrogen assimilation in manure.

3. *B. azophile,* n. sp., which occurs abundantly in fermenting manures, seems to be the chief organism responsible for the increase of nitrogen.

4. The increase in nitrogen, when the organism is grown in a manure extract medium, amounts to from 3 to 5 mgm. per 100 cc. of solution.

5. 28°C. seems to be a favorable temperature for the growth of the nitrogen-assimilating organisms of manure.

[4] Non-chromogenic on some media; chromogenic on bouillon agar and on potato.

NEW DIFFERENTIAL PLATING METHODS FOR B. BIFIDUS (TISSIER) AND B. ACIDOPHILUS (MORO)

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In connection with the study of fecal bacteria the following media and methods have been found useful for the isolation of aciduric forms, especially for quantitative determinations of *B. bifidus* and *B. acidophilus*.

B. BIFIDUS

With laboratory media in common use the isolation of *B. bifidus* from fecal material containing large numbers of *B. coli* is not easy. The preparation of anaerobic plates is necessary, and with glucose agar, which is ordinarily employed, the few colonies of *B. bifidus* developing are generally overshadowed by those of *B. coli*. There is also no well marked difference between the types of colony formation, both surface and deep, of these bacteria in ordinary glucose agar. I have found during the course of a study of the Gram-positive aciduric bacilli of the intestinal tract that solid media prepared with an infusion of beef liver, instead of muscle tissue as a base, is particularly favorable. For the isolation of *B. bifidus* a glucose blood liver agar is employed. In brief, the preparation of this medium is as follows: Cut 500 grams of beef liver in small pieces and add to 1000 cc. of distilled water, boil for two hours in a double boiler, filter through flannel and cotton, and to the filtrate add 10 grams of peptone and 20 grams of agar. Heat in flask in the Arnold for one hour, adjust to the reaction desired and clear with eggs if necessary; to the clear filtrate add 10 grams of glucose and 1 gram of di-potassium phosphate. For *B. bifidus* the medium is

titrated to + 1 to phenolphthalein, and to each 10 cc. about 1 cc. of sterile defibrinated rabbit blood is added, just before the plate is poured. For its optimum development *B. bifidus* requires a certain degree of anaerobiosis, but as will be shown below, it is not an obligate anaerobe, and will grow fairly well aerobically on this medium.

To obtain the reduction of oxygen favorable for the luxuriant development of *B. bifidus*, it has been found advantageous to utilize Nowak's (1908) suggestion for the partial exhaustion of oxygen through the action of a member of the subtiloid group of bacteria. As far as I am aware this method has not been applied hitherto to plate cultures. Perhaps the nearest approach is Horton's (1914) divided test tube device. A plate, however, offers obvious advantages over a tube in that a larger surface of medium is available for seeding and the growth may be readily inspected. The apparatus used by the writer is similar to the anaerobic plate described by Zinsser (1906), except that the lower larger dish contains agar seeded with *B. cereus* (*B. subtilis* would probably prove satisfactory) instead of the pyrogallic acid sodium hydrate mixture.

A Petri dish 10 cm. in diameter and at least 2 cm. high is selected and the glucose blood liver agar is poured into it, taking care that the sides of the dish are kept free from the medium. The dish should be placed in the incubator until the sides are dry and there is no obvious moisture on the surface of the medium, when it may be seeded. This drying is important, as moisture on the sides of the dish may permit contamination by the culture used to absorb the oxygen. Into the cover of a Petri dish about 12 cm. in diameter are next poured about 15 cc. of nutrient 3 per cent agar seeded with the *B. cereus* culture. Before this agar has quite solidified the half-dish containing the seeded liver blood agar medium is inverted and placed in it. The agar in the lower plate on solidifying forms a seal. The apparatus is placed in the incubator in a moist chamber to prevent the exposed agar medium from drying out.

The *B. bifidus* colonies are visible in twenty-four hours, but are not especially distinctive in appearance. After forty-eight

hours incubation, however, they may be readily recognized as raised, more or less *globular*, opaque colonies 1 to 3 mm. in diameter, *buff to reddish brown in color*. In fact the principal novelty in this mode of cultivation lies in the distinctive appearance of the *B. bifidus* colonies (fig. 2), while the method used for obtaining conditions of partial anaerobiosis is of secondary importance. Most strains of *B. acidophilus* form on this medium flat, dingy colonies with a serrated edge, although a few pro-

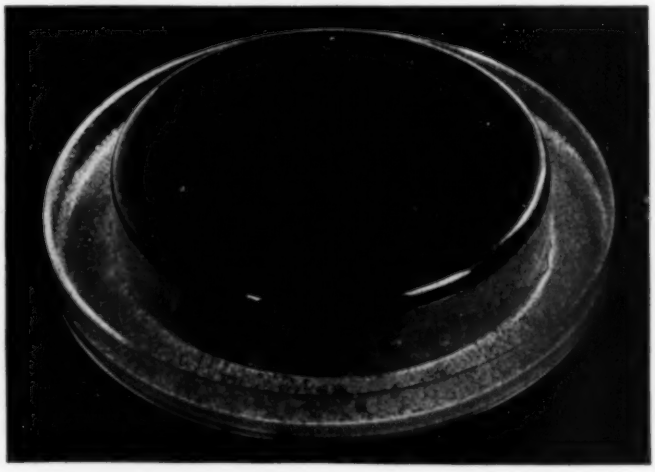

FIG. I. ANAEROBIC PLATE FOR B. BIFIDUS

duce more or less convex whitish or yellowish growths. The *B. coli* colonies are easily distinguished. Hence, if the quantitative count of the viable *B. acidophilus* or *B. coli* has been determined for the specimen, by ascertaining the ratio of the colonies of *B. bifidus* appearing on the plate to those of *B. acidophilus* or *B. coli*, the count for *B. bifidus* may be estimated.

Glucose blood liver agar is so favorable for *B. bifidus* that it will grow on this medium even under aerobic conditions. The colonies in primary plate cultures, however, are generally very small, and appear after forty-eight hours incubation as minute colorless cones. Some strains adapt themselves quickly to

aerobic conditions and give rise to a rather thick staphylococcus-like growth, while others continue to produce a very thin growth on slants of this medium. Both types may be kept alive indefinitely growing on slants under aerobic conditions, if transplants are made about every seven days.

With most strains of *B. bifidus* aerobic growth on liver glucose agar tends to encourage polymorphism, the profuse and variegated branching giving rise at times to coral-like aggregations.

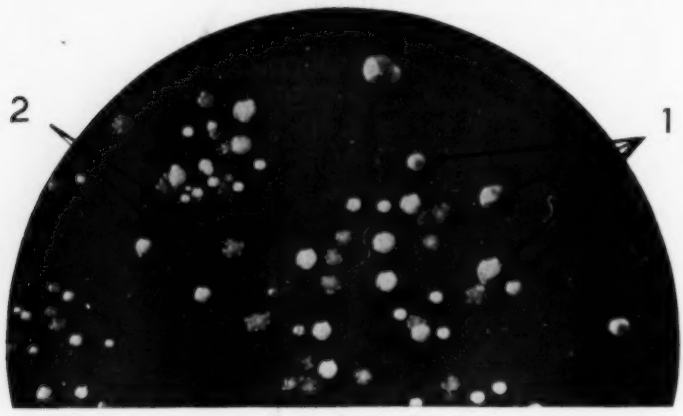

FIG. II. PHOTOGRAPH, SLIGHTLY ENLARGED, OF COLONIES ON A GLUCOSE BLOOD LIVER AGAR PLATE (ANAEROBIC) SEEDED FROM A DOG'S STOOL. 1. B. BIFIDUS COLONIES. 2. B. ACIDOPHILUS COLONIES.

Also, bifid forms are more likely to develop on liver medium than on media prepared with muscle tissue infusion as a base, when cultivated under conditions of partial anaerobiosis.

B. ACIDOPHILUS

Acid glucose agar or oleate glucose agar has generally been recommended heretofore for the cultivation of *B. acidophilus*. I have not found either of these media satisfactory for a quantitative determination of bacilli of this type in fecal specimens. In searching for a substitute it was found that glucose liver agar constitutes a decidedly favorable medium for bacteria of this

type, and furthermore that the typical colony formation facilitates quantitative determinations. The medium is prepared as described above for *B. bifidus*, except that no blood is added to it. *B. acidophilus* will develop on this medium with a range of reaction from neutral to phenolphthalein to +5 acid. A reaction of +3 acid appears to be most favorable, but for fecal work +4 acid is used in order to inhibit the development of streptococci and most strains of *B. coli*.

With a little experience the colonies formed by members of the *B. acidophilus* group may be recognized readily. The type most frequently encountered forms a small fluffy deep colony resembling a fleck of cotton. Certain other strains form globular deep colonies with a serrated border. Acid-tolerating colon bacilli give rise to lenticular deep colonies or sharp pointed triangular ones. Anaerobic plates are not necessary, as with this medium the development of the *B. acidophilus* colony is quite as satisfactory under aerobic as under anaerobic conditions.

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TRICHOMONASIS OF CHICKS: A NEW AND HIGHLY FATAL DISEASE

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During the spring of 1916 a highly fatal disease of chicks was discovered on a ranch near Puyallup, Washington, by Mr. F. W. Breed, poultry expert for the Fischer Flouring Mills Company of Seattle. Nine hundred chicks were hatched at one time from a thrifty stock of white leghorns. Ten days after hatching they developed a disease which rapidly reduced their number to less than one hundred. Sick specimens were brought to the laboratory for the determination of the cause of the trouble. Samples of the feed employed were also furnished to determine whether it might be the source of the disease. Later the same malady was discovered in the large poultry plant of Firland Sanitarium, Seattle, and also at other ranches in the Puget Sound region.

Autopsies revealed no marked lesions and afforded no clue as to the cause. It was natural to assume that the trouble was intestinal in origin, and that it might be either (a) bacterial, (b) protozoal, or (c) fungal. All of these alternatives were tested out on numerous specimens furnished to the laboratory. Bacterial cultures from the organs and heart's blood remained negative when made from killed sick chicks. From specimens which had died *Bact. coli* was isolated; but it never caused disease when fed to sound young chicks six days old.

The intestinal contents were examined for molds, as it appeared quite probable that moldy feed might be the source of the trouble. At the same time culture plates were made from the intestinal contents on agar especially suited to the culture of molds (agar, 20 grams; sucrose, 10 grams; potato water, 200 cc.;

water, 800 cc.). Only three species of molds were found and these were always few in number. In some of the chicks no molds were found. Feeding experiments with the molds isolated (which included *Aspergillus fumigatus*) proved negative, though the faeces showed innumerable colonies of the molds fed. The feed supplied showed few molds and these were of the same species as occurred in the chicks. Further, the feed was given to young control chicks which always throve on it in our laboratory. It appeared certain, therefore, that molds could not be the cause of the difficulty.

Examination of the intestinal contents was also made for coccidia and other protozoa, but they were apparently absent. Blood slides were always negative. For the time being the discovery of the cause of the disease seemed hopeless. At this stage of the investigation it was noticed in one of the autopsies that a caecum was slightly enlarged. The contents of this caecum were examined and revealed the presence of numerous flagellate protozoa. Subsequent examination showed that a single species was present, frequently in overwhelming numbers; that it was not present in healthy stock, or in newly hatched chicks; that healthy chicks kept in cages with sick chicks developed the disease and showed the protozoan in the caeca; and finally that control chicks did not develop the disease when kept under identical conditions. We concluded, therefore, that this protozoan is the cause of the disease.

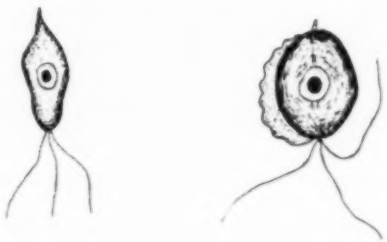
DESCRIPTION OF CHICK DISEASE

The disease apparently attacks young chicks only, for stock that is a month old proves resistant, and chronic cases that reach this age usually recover. The disease appears the second week after hatching, most commonly about the tenth day. Diarrhoea is absent in the early cases, but is present in the chronic stage. In the acute stage the chick may succumb within a day or two, and only the more vigorous birds enter the chronic stage. The chicks droop, huddle together, but do not refuse to eat. The wings drag, eyes are kept closed, and

the heads are pale. The temperature is subnormal and is commonly about 102°. On autopsy no lesions are found save a generally anaemic condition. The caeca may be slightly enlarged and the contents are thin and slimy in proportion to the severity of the disease. The crypts in the mucosa are invaded by the protozoa which form nests in them, frequently in pure culture. If the chicks develop diarrhoea, the protozoan may be found in the intestine and in the voided mucus. Commonly the chick dies in two or three days. Apparently the disease does not appear until late spring and summer, the earlier hatches during the cooler weather escaping it.

DESCRIPTION OF THE ORGANISM

The protozoan is a flagellate belonging to the genus *Trichomonas*. It presents two forms, one pear-shaped, the other globular. The pear-shaped form has a distinct tail, and three flagella situated at the head end, which are nearly the length



1 2

FIG. 1. TRICHOMONAS PULLORUM. × 2000

The pear form or active stage in which the animal moves about by means of flagella, and also shows amoeboid movement.

FIG. 2. TRICHOMONAS PULLORUM. × 2000

The globular form in which the undulating membranes are kept in constant motion.

of the body. The globular form looks like another organism, but the change from one form to the other has repeatedly been observed. In this stage it displays on one side an undulating

membrane which is kept in constant motion; a spine-like process or axostyle is seen at the posterior end. The pear form is the active stage when the organism swims about by means of its flagella and also exhibits distinctly amoeboid movements. This is the only form found in the caecal crypts, but in the lumen it readily assumes the globular type. The protoplasm is finely granular, and the nucleus is situated centrally. The size varies considerably, ranging from 3.5 by 7 μ to 5.5 by 9 μ; the globular form is shorter and broader. The accompanying figures will supplement the description and perhaps clarify it. Since the organism is apparently different from the species described in the available literature (Doflein, 1909; Kolle und Wassermann, 1913), and since Trichomonasis has not been reported in chicks, the name of *Trichomonas pullorum* N. Sp. is proposed by the writer.

SOURCE OF INFECTION

The source of the infection was not definitely determined. Evidently it might come from the soil, the water supply or from "carriers." With incubator chicks it would seem that both the soil and the "carrier" sources can be eliminated, thus leaving the water supply as the probable source.

CONTROL OF THE DISEASE

If the source of the organism is in the water supply its elimination can be effected by boiling or by disinfectants. No opportunity was afforded for trying out these means. By chance another method of control was, however, discovered. One lot of very sick chicks was brought to the laboratory and fed on sour milk. These made a slow recovery. Subsequently healthy chicks were infected in the laboratory and developed the disease; they were then removed from the pens, and their lives saved—if the disease was not too far advanced,—by feeding sour milk. It is quite likely that the lactic acid in the milk acts as an antiseptic or even as a germicide. Possibly other substances could be found equally efficacious, but the use of

sour milk appears to be both rational and convenient, and at the same time it serves as an excellent food.

In closing, the writer desires to acknowledge the receipt of valuable aid concerning the literature, from Dr. C. A. Kofoid, University of California, Dr. E. J. Lund, University of Minnesota, and Dr. A. Eichhorn, Bureau of Animal Industry Washington, D. C.

A STUDY OF THE DIPHTHEROID GROUP OF ORGANISMS WITH SPECIAL REFERENCE TO ITS RELATION TO THE STREPTOCOCCI[1]

PART III. IMMUNOLOGICAL RELATIONS

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IMMUNITY

Natural immunity

Both man and animals possess a relatively high degree of immunity against infection by this group of organisms. Bergey (1904), however, reports spontaneous abscesses in guinea-pigs caused by a diphtheroid, and Klein (1903) proves the causal relationship of this organism to pneumonia in rats. The bacillus of Preiz-Nocard (*B. flavidus*) is pathogenic for most of the lower animals and frequently causes infection among them.

Artificial immunity

Hamilton and Ruediger (1904) were able to develop protective sera against *B. Ruedigeri*. Hall and Stone (1916) report a partial neutralization of the soluble toxin of the Preiz-Nocard organism by diphtheria antitoxin. One minimal ethal dose of their *B. flavidus* toxin required two hundred and fifty times as much diphtheria antitoxin to neutralize it as is required by one minimal lethal dose of the Klebs-Loeffler bacillus toxin. They suggest a group reaction among toxins like the similar reactions which exist among agglutinins. I have been able to develop an antiserum with a filtrate of my strain 1 which pro-

[1] A Thesis for the degree of Doctor of Public Health.

tected against its characteristic effects (Part I). Hamilton (1907) and Rosenow (1915c) report cases which were very markedly benefited by vaccines of diphtheroid bacilli. In Hamilton's cases the vaccines produced a marked rise of the opsonic substances in the serum. On the other hand, vaccines have produced no consistently good results in Hodgkin's disease. Bloomfield gave as many as 10,000,000,000 of the killed bacilli without as much as producing a local reaction. Likewise Townsend's case of diphtheroid cystitis did not seem to be greatly benefited by vaccines.

Phagocytosis

There is very little direct information on this subject, although from the predominance of the purulent conditions produced by this group it is safe to assume a very marked phagocytic response. Experimental infections with my strain 1 developed phagocytosis in some organs but not in others. It was most marked in the joints, while in the gall bladder and kidney almost none could be seen. There was phagocytosis by the splenocytes and in the endothelial-celled thrombi formed in the walls of the blood and lymph vessels of rabbits. Teacher (1915) notes a phagocytosis of *B. flavidus* by neutrophiles present in the damaged placenta of his guinea-pigs.

Toxin formation

It is exceptional for members of this group to form soluble toxins. Ruediger (1903) and Hamilton (1904) have isolated diphtheroid bacilli from both normal and pathological throats which produced a soluble toxin that quickly killed guinea-pigs. Animals were not protected with diphtheria antitoxin, but were protected by a homologous antitoxin. Hektoen was able to demonstrate toxic substances in the cultures of his diphtheroid which produced lesions similiar to those produced by a live culture, although they rarely caused death. Diphtheria antitoxin did not protect. Hall and Stone (1916) report of their *B. flavidus* "1 cc. of unaltered culture kills in fifteen hours. Ten cubic centimeters of the supernatant passed through a Berkefeld filter kills in fifteen hours. One cubic centimeter of toxin-

free culture is fatal in twenty-eight days. The strongest toxin developed killed a guinea-pig in forty-eight hours in a dose of 0.2 cc." Strain 1 of my series produced a soluble toxin in twenty-four hour broth filtrates. It was neutralized by a specific antiserum (Part 1).

Hemolysins

Hall and Stone (1916) report the development of a hemolysin in their cultures, which was not lost on cultivation and was not destroyed by ten minutes boiling. According to Fox's (1915b) tabular reports several of his cultures give positive hemolysis. Strains 13 and 14 of my series gave a slight hemolysis which was lost on continued cultivation.

Agglutination

There have been relatively few agglutination experiments performed with the diphtheroid group. The vast majority of them have attempted to establish a relation between the pseudodiphtheria bacillus (*B. Hoffmannii*) and the true diphtheria bacillus.

Luebowski (1900) developed an immune serum in a goat by using non-virulent diphtheria bacilli. This anti-serum agglutinated twenty-three strains of virulent and two strains of avirulent diphtheria bacilli. The positive dilutions of the serum ranged from 1-80 to 1-160. Three strains of *B. Hoffmannii* were negative.

Lesieur (1901) obtained an anti-serum with diphtheria bacilli and tested 40 strains of Klebs-Loeffler and 30 strains of the pseudodiphtheria organism against it. Of the 70 strains, 29 virulent and 21 avirulent strains reacted negatively, while the remainder were positive. He considers that this is strong evidence in favor of the relationship of certain strains of each series. It is also evidence that the strain variation is quite marked.

Schwoner (1902) obtained a powerful anti-serum in a horse by starting the inoculations with dead cultures and ending with living diphtheria organisms; 50 strains of the Klebs-Loeffler

bacillus reacted positively in dilutions of the serum varying from 500 to 10,000. Reactions with pseudo-diphtheria strains were negative. A goat immunized against a pseudo-diphtheria organism agglutinated only the homologous strain, having no effect on 4 other strains of pseudo-diphtheria bacilli. He concluded from this and similiar experiments that there were many different strains of the Hoffmann bacillus.

Lipstein (1908) developed agglutinating sera in rabbits and found a marked strain specificity. Hamilton (1907) separates three groups of pseudo-diphtheria by means of agglutination reactions. Group I is the common *B. Hoagii*; Group II, *B. flavidus* and Group III the Ruediger bacillus. She says that the agglutinating as well as the lytic sera for these types are specific.

Torrey (1916) has isolated an anaerobe from lymph glands (mostly Hodgkin's) which he believes to be a distinct species, as proved by agglutination experiments.

Teoumin (1913) claims to have separated four groups of pseudo-diphtheria bacilli by agglutination experiments. He developed a 1-400 titre following the third injection. The homologous strains agglutinated, but there was no cross reaction.

Agglutination experiments

For this work I have chosen several strains representative of different subgroups of the diphtheroids. Preliminary experiments in this line correlated with the work of others convinced me that it was not advisable to seek by this criterion an ultimate means of classification. The work done is therefore not as elaborate as it would have been if the phenomenon had been more promising for the purpose for which I wished to employ it.

There are certain definite ends, however, which I thought could be served, and briefly put they are as follows: first, I aimed to get some notion of the relation of my pathogenic strains to the more saphrophytic members of the group; second, to verify my hypothesis regarding the biologic relationship of certain streptococci to certain diphtheroids; and third, to show the bio-

chemical identity of strain 1 in its coccus form with the barred type of the bacillus which gave rise to it.

The technique employed was as follows: The serum dilutions were made in small test tubes, each of which contained 1 cc. of 0.8% salt solution. To these tubes was added 1 cc. of a twenty-four hour broth culture of the organism. The number of organisms in the culture used was held fairly constant by comparison with an arbitrary standard of turbidity. In case the culture was clumped it was put in a mechanical shaker for twenty-four hours. The different dilutions were incubated at 37°C. for one to two hours, placed in the ice-chest over night and final readings taken after eighteen to twenty-four hours. All readings were macroscopic. Dilutions of normal rabbit's serum constituted one set of controls, but in addition a tube of 0.8% NaCl plus 1 cc. of the twenty-four hour broth culture was also used. The various grades of reaction were distinguished by the number of *plus* marks opposite the dilution. *Four plus* indicates complete agglutination with a perfectly clear supernatant liquid: *three plus* indicates a slight turbidity in the supernatant: *two plus* approximately 50 per cent of the organisms present at the bottom of the tube as a sediment: *plus one* indicates only a slight precipitation and *sl* a slight clumping insufficient to cause appreciable precipitation.

Recourse to tables 4 and 5 shows the agglutination reactions of serum 1 with some of the other strains used as antigens. As might be expected the homologous antigen gave a fairly high titre, the reaction being quite noticeable in the 1–2560 dilution, and almost complete in the dilution of 1–1280. The other strains showing a powerful sugar fermentation are strains 14, 16, 11, 2 and 3. It may readily be seen that the bulk of cross-agglutination occurs with these strains, with the exception of strain 11. Strain 12 is *B. xerosis* and T-337 is *B. flavidus*, both of which are mutually related morphologically and fermentatively. They show about the same amount of cross reaction, in low dilution only. Strains 10 and 31 are *B. Hoffmannii*, and give only slightly less cross reaction than strains 10 and 12. Strain 4 is a variant of *B. Hoagii*, which failed to ferment maltose.

It is evident that the active fermenters form a group by themselves, while between the rest of the different types used less demarkation is apparent.

Controls with the various antigens in dilutions of 1–20 normal serum were only slightly positive, and in all cases but one were

TABLE 4

STRAIN NO.	DILUTIONS OF IMMUNE SERUM								IMMUNE SERUM
	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	
J 1	++++	++++	++++	++++	+++	+++	+++	++	I
2	+++	++	+	++	++	+			I
3	++++	++++	+++	++	+++	+	±		I
4	+++	++	++	+					I
10	+++	+++	+	+					I
11	+++	++	+	+					I
12	+++	+++	++	+	−	−	−	−	I
14	++++	+++	++	++	+	+	+		I
16	++++	++++	+++	+++	++	+	+		I
T337	+++	+++	++	++	+				I
31	+++	++	+	+					I

TABLE 5

STRAIN NO.	DILUTIONS OF NORMAL SERUM								CONTROL 0.8% NaCl PLUS 1 CC. CULTURE
	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	
J 1	+								−
2	++								−
3	++++	++	++						−
4	++								−
10	++								−
11	++								−
12	+								−
14	+								−
16	+								−
T337	+								−
31	++								−

completely negative in 1-40 dilutions. Controls with 0.8% NaCl were negative.

It will be necessary to make some explanation regarding immune serum VI. After three injections of the germ-free fil-

trate the animal in question received on December 4, 1915, 1.5 cc. of a twenty-four hour agar culture of live organisms of strain 1 in NaCl solution intravenously. On December 11, 1915, it received 1.75 cc. and on December 15, it received 2 cc. The agglutination titre on December 21 was + + + in a dilution

TABLE 6

STRAIN NO.	DILUTIONS OF IMMUNE SERUM								IMMUNE SERUM NO.
	1-20	1-40	1-80	1-160	1-320	1-640	1-1280	1-2560	
Bile 43..	++++	++++	++++	++++	+++	+++	+++	++	I
Bile 43....	−	−	−	−	−	−	−	−	VI
J1.....	++++	++++	++++	++++	+++	+++	+++	++	I
J1.....	−	−	−	−	−	−	−	−	VI
Lewis streptococcus...	+++	+++	+++	++	++	+			I
Lewis streptococcus...	+++	+++	+++	++	++	++			VI
16.....	+++	++	++	++	++	+++			VI
Joints 43..	++++	++++	++++	++++	+++	+++	+++	++	I
Joints 43..	−	−	−	−	−	−	−	−	VI
J1.....	+	−	−	−	−	−	−	−	β streptococcus
Robert's streptococcus..	+	−	−	−	−	−	−	−	I
Beta streptococcus	+	−	−	−	−	−	−	−	VI
Robert's streptococcus...	+	−	−	−	−	−	−	−	VI
T337.....	+++	+++	++	++					VI
31.....	++	++	++	+					VI

of 1–640. On December 23 the animal received 4 cc. of a forty-eight hour agar culture, but on testing the serum on January 4, 1916, there was absolutely no agglutination with the homologous strain. Just why this rabbit VI which had reacted positively should after another large dose react negatively I am at

loss to explain satisfactorily, although a fuller discussion of this will be entered into later.

It is very curious that, although this serum lost its agglutinating power for the homologenous strain, it still gave cross agglutinating reactions. (See antigens 16, 31, and T337). I was able to make use of this aberrant phenomenon in the demonstration of the identity of the coccic and bacillary forms of the organism, as is shown in table 6.

Recourse to table 1 (Journal of Bacteriology, **2**, 99) shows that rabbit 43 received intravenous injections of strain 1 in bacillary form, and from the gall bladder the organism was recovered in long chains of streptococci or diplostreptococci. In the joints, blood and other organs only diplococci were found. I interpreted this to be an instance of extreme pleomorphism and attempted to test my hypothesis by immune reactions.

If serum VI failed to agglutinate the homologous strain, although it still cross agglutinated related strains, it is obvious that a negative reaction with the organisms recovered from this animal would be strong evidence that the injected bacillus was able to grow in long chains of streptococci. Furthermore, if these streptococcoid forms were agglutinated by homologous serum I to the same extent as the organism which gave rise to this serum, the evidence for the identity of these organisms of such diverse morphology would be still stronger.

Reference to the agglutination tables 4 and 6 will show that just this series of reactions did occur. Bile 43 antigen, which is prepared from the streptococcus form recovered from the bile of the animal injected with the diphtheroid bacillus I, is attacked to the same degree by serum I as is its homologous antigen (I). On the contrary, the reaction with serum VI is entirely negative. The same result is seen with the diplococci recovered from the joints, which antigen is designated "Joints 43." It will also be seen that serum VI still shows cross reactions with antigens 16, T–337 and 31 to about the same extent as was shown by serum I under table 4.

Table 6 also shows some other very interesting reactions. In

addition to the cross reactions of serum VI with other members of the diphtheroid group, a very definite agglutination with the non-hemolytic streptococcus (Lewis) also occurs. Serum I also gives the same reactions to the streptococcus. On the contrary, other types of streptococci give absolutely no cross reactions. The strain "Roberts" which is a hemolyzer of alpha type (*S. viridans*) reacts with neither serum I nor VI. Likewise the streptococcus of beta type of hemolysis (Smith (1915)) gives no reaction with serum VI. Conversely, its immune serum does not react with antigen I, although it reacts feebly with the strain, "Lewis streptococcus." This highly suggestive evidence of the relationship between the streptococcus and the diphtheroid groups should be qualified by stating that it is very probable that the contact is made at certain definite points only through the medium of special strains such as we have here, and does not necessarily imply a relation at other points. For example, the serum labeled beta streptococcus was highly agglutinative for its homologous antigen, but did not agglutinate strain 1 in even a 1–20 dilution. It did, however, give a mild agglutination in 1-40 with the non-hemolytic streptococcus. Likewise the alpha type of streptococcus which is an incomplete hemolyzer gave negative reactions with both serums I and VI.

One sees here a diphtheroid strain not only giving cross reaction with other strains of the same group, but also giving a very definite reaction with a proved streptococcus, and in most cases in greater dilutions than with the diphtheroids. Such crossing is not indiscrimate, however, inasmuch as it does not extend to the representative types of the hemolytic streptococci.

Further experiments with serum VI

I have already referred to the peculiarity developed in this serum, viz., its negative response to the homologous antigen and the positive response to heterologous antigens. It occurred to me that the large doses of living cultures of a fairly active organism might have paralyzed the agglutinating response, although the fact that some antigens did react was rather opposed to such a contention. In case this hypothesis could not be

validated, it occurred to me that the electrical conditions which are supposed to be responsible for the actual agglutination might have been disturbed. To this end I employed a bivalent ion ($CaCl_2$) with the hope that the stronger charge carried by the Ca ion would finally succeed in agglutinating the organisms, provided that they had been actually sensitized (in the sense of Bordet). In place of NaCl as the diluting solution, I used $CaCl_2$, but the results did not vary. I then tried an absorption experiment on serum VI. I mixed a very heavy emulsion of the organism with a 1–40 and 1–160 dilution of the serum, giving ample opportunity for sensitization of the bacilli. On centrifugation, pipetting off the supernatant fluid and

TABLE 7

STRAIN NO.	DILUTIONS OF IMMUNE SERUM								IMMUNE SERUM	DATE
	1–20	1–40	1–80	1–160	1–320	1–640	1–1280	1–2560		
J381...	++++	++++	++++	+++	++	++	++	+	VI freshly drawn	2,11,16
J1.....	+	–	–	–	–	–	–	–	VI	
16.....	+++	++	++	++	++	+++			VI	
J1 in CaCl	+	–	–	–	–	–	–	–	VI	

treating with strain 16, I was able to get the same agglutination as with the serum which had not been exposed to the homologous antigen. This experiment proved that no sensitization of the bacilli took place, and explained the failure of the $CaCl_2$ experiment.

This animal, VI, received its last dose of live organisms on January 1, 1916, and on January 14, 1916, the serum was again tested with the same results. This serum was placed in the icebox and on February 9, 1916, was tested against a bacillary form of strain 1, which had been obtained from the diplococcus form. Surprising as it may seem, this organism (J-381) tested out on February 11, 1916, on serum VI drawn January 14, was agglutinated as strongly as previously by serum I. Moreover, ruling out the effect of the low temperature for three weeks, by

using a fresh specimen of the serum, the same action took place, although it was still refractory to the original strain in diplococcus form.

Controls in 0.8% NaCl and normal serum were all negative. It would seem very probable that some chemical change took place in the organism as a result of the conditions necessary to bring it back to the barred diphtheroid form. There is other evidence for this contention. The bacillary form of this organism is acid-fast to 2 per cent HCl in 20 per cent ethyl alcohol. When converted to the diplococcus form it is non-acid fast, indicating a change which is more than merely one of shape.

My only justification for reporting these results lies first in the fact that I have used this aberrant phenomenon as an aid in demonstrating a rather unusual contention, and second to exhibit one of the freakish occurrences which so many have experienced with the agglutination reaction from time to time.

Complement fixation

There have been but few observations made on the diphtheroid group or on the Klebs-Loeffler bacillus by means of the complement-fixation test. Morse (1912b) reviews what little literature has appeared on the subject, and I shall quote largely from her paper. Lambotte (1901) produced strong specific sera in guinea-pigs for the diphtheria bacillus, a pseudo-diphtheria bacillus from the normal human conjunctiva, and also one from a membrane on a fowl's eye. He gives no description of these bacilli. He shows cross-reactions between the anti-diphtheroid sera and the diphtheria bacillus. Kolmer (1912) has done a convincing piece of work on this subject, using eight strains from diverse members of the diphtheria group, including the Klebs-Loeffler bacillus and *B. Hoffmannii*. He studied several strains of each bacillus representing the different types of bacilli according to Wesbrook's classification, and selected them from different sources. His work tends strongly to show that there is a relationship between *B. Hoffmannii* and the diphtheria bacillus.

On the other hand, Morse is unable to discover any such relation, using the same general method. She says in part: "There are no reciprocal complement-fixation reactions between the typical diphtheria bacillus and representatives of the four species of the diphtheroid group." However, one does not get very definite notions from a careful scrutiny of her experiments. She used only five organisms, and was not successful in developing anti-sera with two of them. Very few of her end results show an appreciable margin between the fixing powers of normal and immune sera. A study of the literature on agglutination work in this group shows that there is probably a great diversity of strains of *B. Hoffmannii* and of the Klebs-Loeffler bacillus. Therefore it would be difficult to draw any conclusions from negative results with so few organisms as Morse used. Priestly (1911) tried to use this reaction for the differentiation of the diphtheria bacillus and diphtheroids, but could develop no satisfactory antigens. He used bacterial extracts and suspensions, but neither proved efficient. Armand-Delille (1908) used diphtheria toxin as antigen, but found wide individual differences in the fixing power of the sera which he could inversely correlate with the signs of anaphylaxis during the immunization. Poujol and Delance (1908) confirmed his results. Cathoire (1911) showed that the serum of healthy carriers fixed complement in the presence of diphtheria toxin.

Hamilton (1907) has developed specific lytic sera for three groups of pseudo-diphtheria bacilli. She specifies the Ruediger bacillus as one, while the others have been identified as *B. Hoagii* and *B. flavidus*, in Morse's classification study.

Technique

1. Antigen preparation. In the preparation of this cardinal component of the system used in complement-fixation work, the method of Swift and Thro (1911) was followed with very slight variations. (a) The organisms were grown on the surface of plain or glucose agar in large Petri dishes for from two to five or six days. The smears were always examined for their purity.

(b) Growth was washed off with sterile 0.85% NaCl and strained if admixed with any of the media. (c) Emulsions were centrifuged at high speed, and organisms washed at least twice with sterile NaCl. The supernatant fluid was pipetted off and the organisms desiccated in vacuo over H_2SO_4 in the icechest (d) The dried bacilli were thoroughly ground with fine sea-sand which had been incinerated to free it of any organic material; then shaken for forty-eight hours in 0.85% NaCl solution containing 0.35% phenol. (5) The débris was then thrown down in a high speed centrifuge and the supernatant fluid stored in dark glass bottles in the icechest. This was kept for the stock extract, dilutions being made up as needed.

2. Complement. Pooled serum of five or six guinea-pigs was diluted to 10 per cent. Graded amounts of a 5 percent solution were titrated against a constant quantity of anti-sheep hemolysin and 5 percent solution of sheep's corpuscles. In the final tests 2 units of complement combined with 0.5 cc. of 1-1000 dilution of anti-sheep amboceptor.

3. Corpuscle suspension. Five percent suspension of sheep's erythrocytes, used in amounts of 0.5 cc. to a tube.

4. Immune sera. Half-grown rabbits were chosen for this purpose. The non-specific fixing powers of a certain percentage of normal rabbit's serum has been shown by Kolmer (1916) and others. Preliminary anti-complementary tests were performed on the rabbits used in this work, and only those selected whose sera reacted negatively with the antigen used. Kolmer has also emphasized another important point in this connection, viz., that any slight tendency a rabbit's serum may have to yield non-specific fixing bodies with bacterial antigens is greatly increased, by exposure to a temperature of 56°C. for a half hour. If the temperature is kept at 62°C. this tendency is much decreased, while it is entirely removed by heating at 70°C. He has also demonstrated that 62°C. does not impair to any significant extent the immune bodies in the serum. Consequently all my sera were inactivated at 62°C., instead of 55°C.

It required from four to six weekly injections of moderately large doses of the living organisms to develop the fixing power

that I desired in the sera. The organisms were grown on glucose agar.

It was the original intention to develop eight immune sera with as many different strains of organisms which seemed fairly representative of the series studied. All animals yielded a satisfactory serum except the ones receiving strain T337. These developed a progressive loss of weight until one-half of their original weight had been lost. Their sera yielded no immune bodies, and they usually died at this stage. This experience is in accord with that of Hamilton (1907) when working with *B. flavidus*.

Results of experiments

The anti-sheep hemolytic system was used in all the tests. After determining the anti-complementary dose of the antigen it was used in one-fourth to one-sixth of that quantity. The preliminary test for the strength of the components of the hemolytic system was performed as follows: 0.5 cc. of a 5 percent suspension of fresh sheep's corpuscles was added to 0.5 cc. of an amboceptor diluted 1–1000 in NaCl. A 5 percent solution of pooled complement was used in graduated amounts, from 0.5 cc. to 0.1 cc. NaCl solution was added to bring the volume to 2.5 cc. The time limit was thirty minutes. Simultaneously an amboceptor control was run as follows: 0.5 cc. of the corpuscles plus 1 cc. of 5 percent complement with graded amounts of amboceptor from 0.4 to 0.1 cc. In case complete hemolysis did not develop in the last two tubes, the amboceptor was not considered to be too strong.

Complement and antigen were mixed with NaCl to make a total volume of 1.5 cc., added to each tube containing the immune serum, and incubated for forty minutes. Simultaneously the necessary quantity of sheep's cells and amboceptor were mixed and incubated for forty minutes for sensitization. At the end of this time 1 cc. of this mixture was added to each tube of the complex and readings taken at the end of one hour.

In the fixation work shown in the following tables (8 to 18 inclusive) the amount of immune serum used was at no time

greater than 0.1 cc., the reason for this procedure being given more in detail on page 470. An inspection of the tables will show that cross-fixation failed in many instances when using 0.1 cc. of the serum. With antigen T337 almost none of the sera gave cross fixation. Antigen 12 was little better. It might be argued that strain T337 had absolutely no relation to the other diphtheroid races. As a matter of fact such is not the case, and in order that misconceptions of this sort may not arise, and that the true significance of this negative reaction may be interpreted, I concluded to carry the experiments further.

When increasing amounts of immune serum are employed (table 21) the antigen T337 exhibits very definite cross-fixation with several strains, and there is still a margin between a normal and immune serum. Strain T337 by its fixation reaction would seem farther removed from most of the diphtheroid strains than they are from one another; but it is not removed in the direction of the streptococcus group, as is strain 1, but, from a consideration of its cultural and other characteristics, we have good reason for believing, in the direction of the Klebs-Loeffler bacillus itself.

That the use of these increased amounts of immune serum shown in table 21 is legitimate, can be seen from the results in table 19; it is evident that antigen T337, as well as several others, gives no reaction with normal heated serum until it is used in quantities of about 0.25 cc., and that then the fixation is only slight. This circumstance permits the use of immune sera in amounts just short of those which will give fixation with normal sera. Accordingly in table 21 fixation experiments have been performed using graded amounts of immune serum, until 0.25 cc. is reached. The sera and antigens which gave no interreaction in the tables 8 to 18 were naturally selected.

TABLE 8

Table showing fixing and anticomplementary power of antigens

NORMAL SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	T-337	X-323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.1	0.1	C.H.	C.H.	C.H.	S.H.I.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.15	C.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.2	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.3	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	I.H.	C.H.	S.I.H.	M.I.H.	C.H.
0.1	0.35	M.I.H.	C.H.	C.H.	I.H.	C.H.	C.H.	I.H.	C.H.	M.I.H.	I.H.	M.I.H.
0.1	0.4	I.H.	C.H.	C.H.	I.H.	S.I.H.	M.I.H.	I.H.	M.I.H.	I.H.	I.H.	I.H.
0.1	0.5	I.H.	M.I.H.	M.I.H.	I.H.	M.I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.
Antigenic unit...		0.07 cc.	0.09	0.08	0.02	0.08	0.07	0.03	0.07	0.055	0.05	0.065
						Immune serum						
0.1	0.01	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.				C.H.
0.1	0.03	I.H.	I.H.	I.H.	S.I.H.	I.H.	I.H.	I.H.				M.I.H.
0.1	0.05	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.
0.1	0.07	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.
0.1	0.09	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.
0.1	0.12	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.
0.1	0.15	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.

The following abbreviations will be used in all tables to record reactions: C.H., complete hemolysis; S.I.H., slight inhibition of hemolysis, 10 to 20 per cent; M.I.H., marked inhibition of hemolysis, 75 to 90 per cent; P.H., partial hemolysis, approximately 40 to 50 per cent; I.H., complete inhibition of hemolysis.

TABLE 9

Immune serum 1

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	T-337	X-323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	M.I.H.	C.H.	C.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.03	com-	I.H.	C.H.	C.H.	M.I.H.	P.H.	I.H.	M.I.H.	C.H.	M.I.H.	C.H.	C.H.
0.05	ple-	I.H.	C.H.	C.H.	I.H.	M.I.H.	I.H.	M.I.H.	C.H.	I.H.	C.H.	C.H.
0.01	men	I.H.	C.H.	C.H.	I.H.	M.I.H.	I.H.	M.I.H.	C.H.	I.H.	C.H.	C.H.
0.01	tary	I.H.	S.I.H.	C.H.	I.H.	I.H.	I.H.	M.I.H.	C.H.	I.H.	C.H.	C.H.
	dose											

TABLE 10

Immune serum 2

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	T-337	X-323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	S.I.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.03	com-	C.H.	I.H.	I.H.	S.I.H.	M.I.H.	S.I.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.05	ple-	C.H.	I.H.	I.H.	I.H.	S.I.H.	I.H.	S.I.H.	C.H.	P.H.	C.H.	C.H.
0.07	men-	C.H.	I.H.	I.H.	I.H.	M.I.H.	I.H.	S.I.H.	C.H.	P.H.	C.H.	C.H.
0.1	tary	C.H.	I.H.	I.H.	I.H.	M.I.H.	I.H.	P.H.	C.H.	P.H.	C.H.	S.I.H
	dose											

TABLE 11

Immune serum 34

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	S.I.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03	com-	C.H.	I.H.	I.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05	ple-	C.H.	I.H.	I.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.07	men-	C.H.	I.H.	I.H.	C.H.	C.H.	I.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.1	tary dose	S.I.H.	I.H.	I.H.	C.H.	C.H.	I.H.	S.I.H.	C.H.	S.I.H.	C.H.	S.I.H.

TABLE 12

Immune serum 32

IMMUNE SEPUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03	com-	C.H.	S.I.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05	ple-	S.I.H.	M.I.H.	C.H.	I.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.07	men-	P.H.	M.I.H.	M.I.H.	I.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	tary dose	P.H.	S.I.H.	I.H.	I.H.	I.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.

TABLE 13

Immune serum 31

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03	com-	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05	ple-	S.I.H.	M.I.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.07	men-	I.H.	M.I.H.	C.H.	C.H.	I.H.	S.I.H.	C.H.	S.I.H.	S.I.H.	C.H.	S.I.H.
0.1	tary dose	I.H.	M.I.H.	C.H.	C.H.	I.H.	S.I.H.	C.H.	M.I.H.	S.I.H.	C.H.	M.I.H.

TABLE 14

Immune serum 3

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	I.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	M.I.H.	I.H.	I.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03	com-	I.H.	I.H.	I.H.	S.I.H.	C.H.	I.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.
0.05	ple-	I.H.	I.H.	I.H.	M.I.H.	C.H.	I.H.	S.I.H.	S.I.H.	C.H.	C.H.	C.H.
0.07	men-	I.H.	I.H.	I.H.	M.I.H.	C.H.	I.H.	S.I.H.	M.I.H.	S.I.H.	P.H.	C.H.
0.1	tary dose	I.H.	I.H.	I.H.	M.I.H.	C.H.	I.H.	P.H.	M.I.H.	M.I.H.	M.I.H.	S.I.H.

TABLE 15

Immune serum streptococcus 20 (Lewis)

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.
0.03	com-	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.
0.05	ple-	P.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.
0.07	men-	P.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	S.I.H.	P.H.	C.H.
0.1	tary dose	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	M.I.H.	M.I.H.	C.H.

TABLE 16

Immune serum 12

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.
0.03	com-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.
0.05	ple-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	I.H.
0.07	men-	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	P.H.	C.H.	C.H.	I.H.
0.1	tary dose	C.H.	C.H.	P.H.	P.H.	P.H.	S.I.H.	C.H.	M.I.H.	S.I.H.	C.H.	I.H.

TABLE 17

Antigen 1 against various sera

IMMUNE SERUM	AMOUNT ANTIGEN	SERUM 1	SERUM 2	34	32	31	3	LEWIS STREPTOCOCCUS 20	12
cc.	*cc.*								
0.005	1–16	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03	comple-	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05	mentary	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	P.H.	C.H.
0.07	dose	I.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	M.I.H.	C.H.
0.1		I.H.	C.H.	C.H.	M.I.H.	C.H.	M.I.H.	I.H.	C.H.

17A

Antigen from bile of rabbit 43

IMMUNE SERUM	AMOUNT ANTIGEN	SERUM 1
0.005	1–6	C.H.
0.01	Anti-	M.I.H.
0.03	comple-	I.H.
0.05	mentary	I.H.
0.07	dose	I.H.
0.1		I.H.

TABLE 18

Antigen 1 against various sera

IMMUNE SERA	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	H. STREPTOCOCCUS		
cc.	*cc.*										
0.005	1–16	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		
0.01	Anti-com-	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		
0.03	plemen-	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		
0.05	tary dose	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		
0.07		I.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	M.I.H.	C.H.		
0.1		I.H.	C.H.	C.H.	M.I.H.	C.H.	M.I.H.	M.I.H.	C.H.		

TABLE 19

Concentration of fixing bodies in normal sera

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 34	T-337	2	3
cc.	*cc.*				
0.13	1–4	C.H.	C.H.	C.H.	C.H.
0.18	Anti-com-	C.H.	C.H.	C.H.	C.H.
0.25	plementary dose	S.I.H.	S.I.H.	C.H.	P.I.H.

TABLE 20

Antigen 2 against sera

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	20	
cc.	*cc.*			
0.13	1–6	C.H.	S.I.H.	
0.18	Anti-com-	C.H.	M.I.	
0.25	plementary dose	P.H.	P.H.	

TABLE 21

Antigen T-337 against sera using 1–4 anticomplementary dose

IMMUNE SERUM	1	2	34	32	31	3	20			
cc.										
0.13	P.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.			
0.18	P.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.			
0.25	M.I.H.	C.H.	M.I.H.	C.H.	S.I.H.	S.I.H.	C.H.			

TABLE 22

Antigen 34

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	31	20
cc.	*cc.*			
0.13	1–6	P.H.	S.I.H.	C.H.
0.18	Anti-comple-	P.H.	P.H.	C.H.
0.25	mentary dose	M.I.H.	M.I.H.	C.H.

TABLE 23

Antigen 3

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	32	20
cc.	*cc.*			
0.13	1–6	M.I.H.	M.I.H.	C.H.
0.18	Anti-comple-	M.I.H.	I.H.	C.H.
0.25	mentary dose	M.I.H.	I.H.	P.H.

TABLE 24

Staphylococcus antigen against various sera

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	3	31	32	34	NORMAL SERUM		
cc.	*cc.*									
0.05	1–6	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	0.1	C.H.	
0.07	Anti-comple-	I.H.	C.H.	I.H.	C.H.	I.H.	C.H.	0.2	C.H.	
0.1	mentary dose	I.H.	C.H.	I.H.	C.H.	I.H.	C.H.	0.25	C.H.	
0.05	1–12	I.H.	C.H.	I.H.	C.H.	C.H.	C.H.	0.1	C.H.	
0.05	Anti-comple-	I.H.	C.H.	I.H.	C.H.	C.H.	C.H.	0.2	C.H.	
0.1	mentary dose	I.H.	C.H.	I.H.	C.H.	M.I.H.	C.H.	0.25	C.H.	

Control

That the cross fixation of diphtheroid sera is not indiscriminate can be seen by the following table, where a heterologous antigen in the form of the alcoholic extract of beef heart was employed. The results are uniformly negative.

TABLE 25

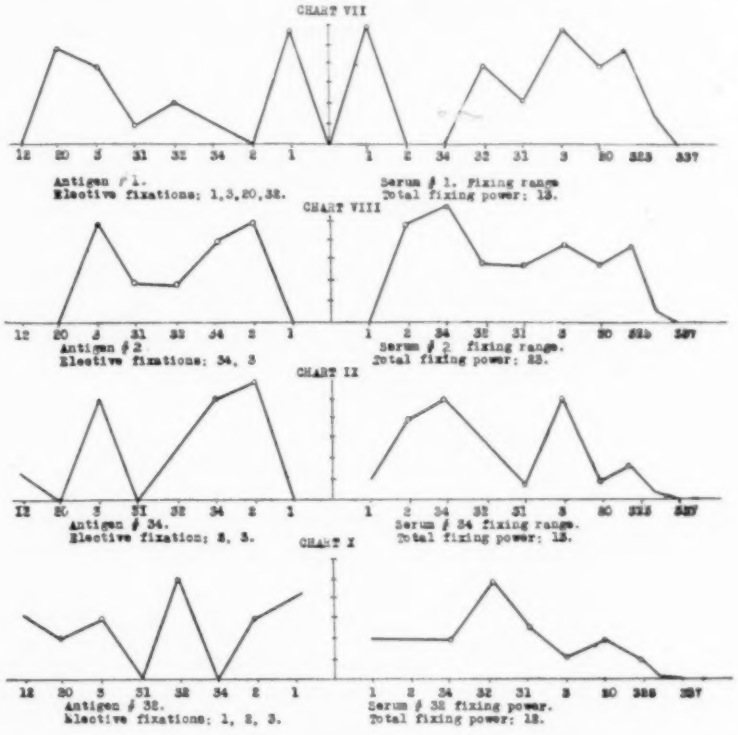
Antigen. Alcoholic extract of beef heart, reinforced with cholesterin. Antigen with syphilitic antibody in 0.1 cc.; is anti-complementary in amounts of 0.5 cc.

SERUM	AMOUNT ANTIGEN	1	2	34	32	31	30	3
0.05	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.09	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.15	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.25	0.1	C.H.	C.H.	C.H.	S.I.H.	C.H.	S.I.H.	C.H.

The curves which follow are attempts to formulate in a roughly quantitative manner the results shown in the preceding tables. This formulation will be presented in two phases. One curve will show the "fixing range" of the various sera toward representative antigens (right hand curve); the other will show the specific direction in which the fixing power is spent, or the so-called "elective fixations." It is obvious that these two curves are to a great extent mutual reciprocals, and the above ideas are not expressed in a segregated way by either curve alone. But since a serum involves concentration of immune bodies, and since an antigen is used mainly for specific reaction, it would seem that there is enough individualization to merit a formulation on the above basis, even though one must necessarily be expressed somewhat in terms of the other.

To this end the concentration of the immune bodies in the homologous sera was developed to as near the same titre as possible, and it will be seen that the dilutions of immune sera in the various tests were held constant and were not carried beyond 0.1 cc. There was a definite reason for using serum concentrations within a certain range. My principal object in the tests

was to determine the interrelations of various members of the group, as well as their relation to the streptococci. By using the immune serum in low concentration it was possible to bring out shades of reaction that would not have been obtained had I used sera in amounts close to the fixation level of normal sera. For this reason it will not be possible to make sweeping generaliza-



tions from these experiments as, e.g., in regard to the relation of the diphtheria bacillus to *B. Hoffmannii* which had an indirect relation to this study.

In charts VII–XIV two curves have been placed on each horizontal axis, which is divided in the center by a vertical axis. The one to the right may be called the serum curve and the one

to the left the corresponding antigen curve. The figures on the right horizontal axis represent the various antigens against which the serum was titrated, while those on the left represent the serum corresponding to the antigens against which a single antigen was titrated. The six graduations on the vertical axis represent roughly the different dilutions of sera used against the

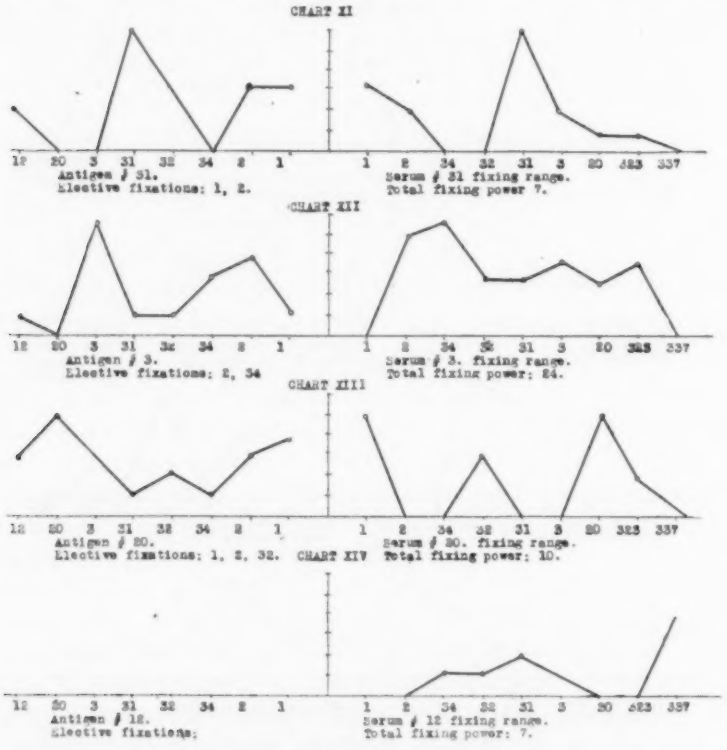
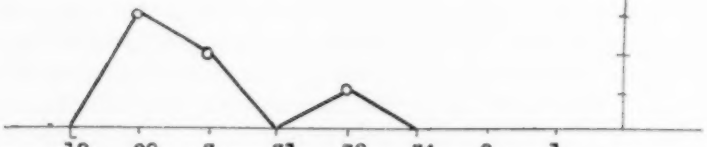


CHART XI
12 20 3 31 32 34 2 1
Antigen # 31.
Elective fixations: 1, 2.
1 2 34 32 31 3 20 323 337
Serum # 31 fixing range.
Total fixing power 7.
CHART XII
12 20 3 31 32 34 2 1
Antigen # 3.
Elective fixations: 2, 34
1 2 34 32 31 3 20 323 337
Serum # 3. fixing range.
Total fixing power: 24.
CHART XIII
12 20 3 31 32 34 2 1
Antigen # 20.
Elective fixations: 1, 2, 32.
1 2 34 32 31 3 20 323 337
Serum # 20. fixing range.
Total fixing power: 10.
CHART XIV
12 20 3 31 32 34 2 1
Antigen # 12.
Elective fixations:
1 2 34 32 31 3 20 323 337
Serum # 12 fixing range.
Total fixing power: 7.

various antigens. They do not in every instance record with precision what is found in the corresponding table, since the degrees of hemolysis expressed there cannot be read in per cent.

The term "fixing range" means simply the *number* of antigens which would be fixed by a certain serum, while total fixing power means an addition of all the fixations recorded which were ex-

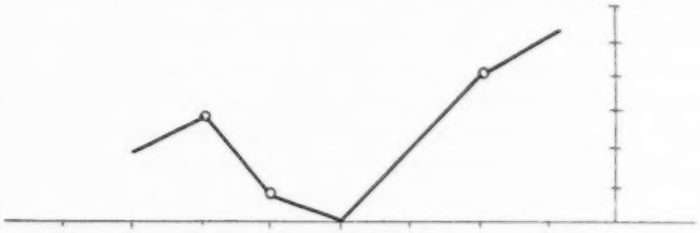
CHART XV



12 20 3 31 32 34 2 1

Antigen # 1. 1/16 anticomplementary dose.
Elective fixations: 20, 3, 32.

CHART XVI



12 20 3 31 32 34 2 1

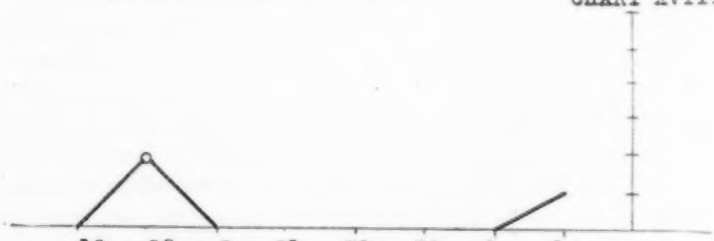
Antigen x-323.
Elective fixations: 1, 2, 3.

CHART XVII

12 20 3 31 32 34 2 1

Antigen T-337.
Elective fixations: 1, 12.

CHART XVIII



12 20 3 31 32 34 2 1

Antigen hemolytic streptococcus.
Elective fixation: 20.

pressed numerically. Each gradation on the vertical axis was given a value of 1, and on this basis the fixing power of a serum ranged from 1 to 6. The latter figure usually expressed the fixation power of the homologous serum. Certain antigens seemed to have a decided reaction with certain sera, and this was expressed by using the term "elective fixation." It is practically synonomous with cross fixation and could be called maximum cross fixation.

Discussion

We will consider first the evidence of relationship between the streptococcus and diphtheroid groups, and the part played by strain 1 in its demonstration. It will be seen from the curves that the streptococcus antigen 20 shows definite cross-fixation with the sera of strains 1, 2, and 3, but practically none at all with sera 31 and 34. In addition, serum 20 gives a strong cross reaction with antigen 1 and X-323, although the reciprocal reaction with sera 2 and 3 is not so apparent. This points to definite relation between a moderately pathogenic, non-hemolytic streptococcus, and certain diphtheroid strains. As the tables will show, this reaction is most marked in strain 1.

In order to subject this relationship to a more severe test it was decided to use a minimum quantity of the antigen. Accordingly, 1–16 instead of 1–6 of the anti-complementary dose of strain 1 was employed. Reference to table No. 18 shows the results. With the exception of the homologous serum, the streptococcus 20 gave more fixation than any other strain. In fact the rest were all negative except strains 32 and 3. This indicates a closer relation between this streptococcus and strain 1 than between the latter and all the rest of the diphtheroids used in these tests. Correlated with the other characteristics of the other two strains, it places this one in an intermediate position between the two groups and shows definitely the connection between them. However, it is probable that this contact between the groups only takes place at certain points, since it is seen that the hemolytic streptococcus antigen (H. strep.) gives no cross reaction with any of the diphtheroid strains

and only gives a slight amount with the non-hemolytic streptococcus 20.

Kolmer (1912) finds no cross-reactions between the diphtheria group and the streptococci. He studied 8 strains, with a view to showing a relation between the Klebs-Loeffler bacillus and *B. Hoffmannii*. His various antigens were tested against an anti-streptococcus serum made from scarlet fever streptococci. These were probably hemolytic, which might easily explain the negative fixation. Even so, this particular table does not appear to be controlled, inasmuch as there is no record of a normal serum being used. Since the immune serum crossfixes in doses of 0.15 cc. and over, it would be essential to show that a normal horse serum in this small dose would also react positively to the various antigens employed.

It may next be asked what light the tests throw on the interrelations of the diphtheroid subgroups. Scrutiny of the curves of the various sera would lead one to infer that strains 1, 2, and 3 have a similar fixing range and total fixing power. Respectively, the total fixing power for these sera is 24, 23, and 24. These might be considered as being members of the same group. This idea is further substantiated when the elective fixations of the three sera are examined. It is seen that a very close relation seems to exist between 1 and 3, and 2 and 3, but it is not so marked between 1 and 2.

It is also very noteworthy that antigen T-337 gives practically no fixation with any other serum except strain 12. Likewise the serum of strain 12 has a total fixing power of but 7, which is slightly less than that of *B. Hoffmannii*. Strain T-337 represents the subgroup *B. flavidus;* and strain 12, *B. xerosis* (Morse). Hamilton (1907) believes the former is closely related to the Klebs-Loeffler bacillus, while the latter has often been described as an avirulent diphtheria bacillus. Thus these organisms are removed biologically from the other diphtheroids, but not in the direction of the streptococcus group. The complete relation has been discussed at length under "Classification" (Journal of Bacteriology, **2**, 299). Morse (1912b) also showed an intimate relation between *B. flavidus* and *B. xerosis*. Table 21

shows its relation to *B. Hoagii*, which has also been noted by Morse, though she conceives them to be much more closely related than my experiments would indicate.

Strain 31 (*B. Hoffmannii*) has a total fixing power of only 8, although it has cross-fixations with the streptococcus group. Strains 34 and 32 have about the same total fixing power, although the former is closely related to the pathogenic group, and the latter to the saprophytic group. From table 24, it would seem that this group has a relation at certain points to the staphylococci, as sera 1 and 32 give definite cross reactions with a staphylococcus antigen, even in one-twelfth of the anti-complementary dose.

Summary

The complement-fixation reactions show that:

1. The diphtheroid group is a very complex one, the various subgroups of which may be widely removed from each other.
2. There exists an unequivocal relation between this group and the streptococci at certain points, at which points the bonds uniting the intermediate diphtheroid strain to the streptococcus group may be much firmer than those uniting it to its own group.
3. The same thing appears to be true to a much lesser degree, with staphylococci.
4. It would not be surprising if the diphtheroid group might impinge upon other more pathogenic organisms, as is connoted by the study of De Witt's strain (1912), suggesting as it does a relationship with *B. proteus*.

General pathology of diphtheroid infections

That the virulence of this group of organisms is usually not high is suggested by the fact that some member of it has been isolated repeatedly from most all of the organs of the body, whether normal or in a state of disease. When one says that most diphtheroid invasions are of the nature of sub-infections, little more comment need be made. However, there sometimes arise acute inflammatory processes which may at times be a

serious menace to life. Hodgkin's disease may begin as an acute inflammation of the lymph glands, and Bunting believes that a diphtheroid in such instances gives rise to an exudation composed of fibrin, neutrophiles, mononuclears, plasma-cells and eosinophiles. Small areas of necrosis are also produced. He claims to have reproduced the acute or toxic form of this disease in monkeys. Other examples of purulent exudation occur in otitis media which will be described under the head of "Pathogenicity" De Witt (1912), Cave (1912) and many others have described a membranous exudation arising usually from the mucosa of the throat. In De Witt's case the vagina also was involved, not only with the membrane but with an indolent ulceration as well. These organisms have produced albuminuria and sepsis (strain 2). Chronic inflammation may be produced. The interstitial changes in the lung from which strain 1 was isolated were of long standing. The fibrosis and endothelial-celled hyperplasia of Hodgkin's disease may prove to be caused by a diphtheroid.

Special pathology. It will be necessary only to refer briefly to the special pathology which has been produced by diphtheroids, as the conditions will be mentioned under the head of "Pathogenicity." Tonsillitis (Hamilton, 1904), chalazion, acute and chronic purulent otitis media (Hamilton, 1907), arthritis (Rosenow, 1915a), caseous and ulcerative lymphangitis in cattle (Hall and Stone, 1916), erythema nodosum (Rosenow, 1915b), broncho-pneumonia and interstitial pneumonia (Rosenow, 1914b), ulcerative cystitis (Rosenow, 1915c) and an epizootic of infective abortion in guinea-pigs are the principal examples (Teacher, 1915).

Experimental pathology. Local abscess at the site of injection has been reported by many observers. Chalazion has been produced by Eyre (1896); hepatic cirrhosis by Hektoen (1901); nephritis by Dick (1915); sepsis by Hektoen (ibid), Hamilton (1904), Hall and Stone (1916), and by Rosenow (1914); paralysis by Hamilton (1904); orchitis by Hall and Stone (1916); broncho-pneumonia, myocarditis, pericarditis, erythema nodosum, cholecystitis and acute pancreatitis by Rosenow (1914b).

PATHOGENICITY

The diphtheroid group has not been taken seriously, from the standpoint of pathogenicity, by the majority of observers, although a wide divergence of opinion exists. Such an attitude has not conduced to thorough investigation and study of strains which had a suggestive connection with pathological processes. Particularly, since the claims which have been made for the diphtheroid etiology of Hodgkin's disease, those interested in the subject have usually taken a decided stand in the direction indicated by their judgment and experience. Generally speaking, the tissue pathologists pure and simple are strongly opposed to the theory of diphtheroid pathogenicity, inasmuch as they have considered Hodgkin's disease a tumor. The biologists and bacteriologists are more impressed with the pathologic possibilities of the group.

Fox (1915a) has recently made a review of the literature on this subject, and comes to the conclusion that there are only four established cases of diphtheroid infection on record. He insists strongly that the immunity reactions are the only ultimate criteria by which one is able to establish such relation, especially in view of our inability to reproduce the corresponding pathology in animals. He reviews twenty cases of a suppurative nature from the records of the Pepper Clinical Laboratory in which some type of this organism was found, and believes that endocarditis was produced in one which gave positive immunity reactions. He does not state whether immunity reactions were performed on the others. He reports three cases of pseudodiphtheritic angina which were not benefited by antitoxin. No immunity tests were recorded with these cases, although he believes the condition was caused by a diphtheroid. He concludes his paper by saying "that excepting these three cases of his own, there are but four cases of diphtheroid infection on record."

In another paper on lymph gland diphtheroids, Fox (1915b) considers the cultural, morphological and biological characteristics of various diphtheroid strains isolated from lymph glands of Hodgkins' disease and other conditions, especially enlarged

joints. In conjunction with his own strains he studied some of those from Rosenow and Kolmer. He concludes that there is no uniformity in the biology and morphology of races isolated by three observers from clinical and pathological Hodgkin's glands. Diphtheroid rods similar in biology and morphology to those found in Hodgkin's disease may be found in enlarged glands in cases of atrophic arthritis and other conditions.

I have already taken exception to the method which he employed for his sugar reactions, and have given my reasons for so doing under the head of "Fermentation" (Journal of Bacteriology, **2**, 289). Close scrutiny of his morphological observations would seem to show that he varies indiscriminately the numerous factors on which a constant morphology depends. The drawings showing marked pleomorphism and staining variations are made from cultures incubated for varying lengths of time on Bordet's medium, Loeffler's blood-serum, agar, and broth arranged in such a way that there is little opportunity for drawing parallels. The advocates of morphological strain constancy all emphasize the fact that the various conditions of the experiment must be held constant in order to obtain constant results (Williams, 1902).

Wade and Harris (1915) also review the literature and come to practically the same conclusion as Fox. They believe that the diphtheroids should be regarded merely as contaminators, or at best as secondary invaders. He cites a very striking positive case of Hektoen's, and in light of it says that a few strains *may* be pathogenic. His paper is entitled "The Wide-Spread Distribution of Diphtheroids and their Occurence in Various Lesions of the Human Tissues." Since he is able to find some type or other of diphtheroids in almost every tissue examined, whether normal or diseased, he cannot attribute much pathological significance to the group.

Torrey (1916) has very recently published a study of the lymph gland bacteria. He cultured thirty strictly lymph gland conditions and ten conditions where the lymph glands were secondarily involved. He recovers one type or other of diphtheroid from twenty-two cases. His virulence experiments with

monkeys are all negative. He was able to produce nothing but transitory glandular enlargements.

Torrey recovered the pleomorphic granular type of diphtheroid in only two cases out of ten of Hodgkin's disease. He is very skeptical concerning the relation of a diphtheroid to Hodgkin's disease for the following reasons:

1. The identity of various strains has not been established.
2. Pleomorphism is common to other members of this group.
3. The description of colony formation has not been uniform.
4. There is no report of carbohydrate action of the various strains.
5. There are many types of diphtheroids reported from single cases of Hodgkin's.
6. The lack of serological evidence advanced.

In addition he says that these pleomorphic and granular forms have been isolated from a diversity of conditions. Furthermore he has investigated one of Bunting's strains from Hodgkin's glands and two of Rosenow's strains and finds no similarity of sugar or serum reactions. The morphological and cultural features also are unrelated.

He believes that considerable caution should be observed even when accepting serological results as evidence of the causal relation of an organism to a malignant growth. This surmise came as the result of his isolation of an anaerobic diphtheroid which bore no relation to the condition in which it was found, but gave strongly positive serum reactions with sarcoma and Hodgkin's cases. His critical remarks are very consistent and his position well taken.

Bloomfield (1915) also makes a study of the "Bacterial Flora of Lymphatic Glands." Examination of seven normal lymph glands showed positive cultural results in two-thirds of the cases, while a still larger per cent obtained in examination of twenty-three cases of diseased glands. He tested the virulence of 14 strains on rabbits, mice and guinea-pigs, with negative results. He believed that the organisms were parasitic but non-pathogenic. Immune reactions on patient's sera were negative except with two of the anaerobic strains. Vaccines had no effect

even in doses of 10,000,000,000 bacteria. He reports one interesting case of atypical chronic arthritis with splenomegaly, general adenitis and leucopenia. He isolated in large numbers many small Gram-positive diplobacilli; they were non-virulent for small animals, but agglutinated and fixed complement with the patient's serum. He comes to the conclusion that a great deal of conservatism should be used in passing judgment on the diphtheroid etiology of Hodgkin's disease.

I shall now briefly review those instances which I have been able to find in the literature that prove diphtheroid pathogenicity or are highly suggestive of it. At the head of this list one must place the results of Hamilton, who has always insisted that these organisms played a much larger part in disease than ever has been accredited them. Her well-reported studies are illuminating. I shall treat this subject regionally.

Eye, ear, nose and throat

In one paper Dr. Hamilton (1904) studied the virulent group of diphtheroids recovered mostly from throats of scarlet fever, measles and tonsillitis subjects. Out of 31 strains, 14 were pathogenic. The group divided itself into two classes, using as a criterion the pathology in guinea-pigs.

Class I. Intraperitoneal injection of amounts of the broth cultures equivalent to 1 per cent of the body weight of the animal gave rise to local edema, hemorrhage, peritoneal effusion, emaciation and death. Cultures from the injection site and effusion were positive. One animal died of typical post-diphtheritic paralysis in four days.

Class II. The animals died in eighteen to twenty-four hours. There was no rigor or infiltration at the injection site. Animals died of septicemia.

Eight of these 14 strains belong to Class II and are Ruediger diphtheroid bacilli. They produce a soluble toxin which is neutralized by its own antitoxin but not by diphtheria antitoxin. The other strains were pseudo-diphtheria culturally, but fermented glucose with gas. They belonged to Class I, and the animals

were protected by neither Ruediger antiserum nor diphtheria antitoxin. Here one finds at least fourteen instances of diphtheroid pathogenicity, proven by the animal test as well as serologically.

In addition the organisms in Cases number 1, 4, 9, and 13 of her series were very probably pathogenic for man, although no results were obtained in animals. Case 1 was one of acute articular rheumatism in which the organisms were found in pure culture on the tonsils. Zarniko (1889) reports a similar instance. In Case 4 a diphtheroid was obtained in pure culture from a tonsillar abscess. In Case 9 the diphtheroid was the predominating one in a severe tonsillitis of a child with an atypical rash, while in Case 13 (scarlet fever) the organism was obtained in pure culture from an aural discharge.

In another communication Hamilton (1907), she gives the results of a study of 52 cases of suppuration in various parts of the body, and 142 cases of purulent otitis media.

TABLE 26

	NUMBER OF CASES	NUMBER WITH DIPHTHERIA	PER CENT WITH DIPHTHERIA
1. Acute scarlatinal otitis media	43	31	72
2. Chronic scarlatinal otitis media	9	5	55
3. Acute non-scarlatinal otitis media	19	4	21
4. Chronic non-scarlatinal otitis media	71	11	15
5. Miscellaneous suppurations	52	11	21

In nine cases of the acute scarlatinal otitis media, the diphtheroid was found in pure culture, and in sixteen cases only a few colonies of other organisms were present. Two chronic cases with mastoid complications yielded the organism in pure culture from both locations. In the non-scarlatinal otitis media, it was found in a much smaller percentage of cases and usually mixed with many pyogenic cocci. The percentage difference between scarlatinal otitis media and between non-scarlatinal and other forms of suppuration is very marked and leads Hamilton to ask "If it is only an inhabitant of the aural tract, as has been claimed, why is it that it is found almost to the exclusion of other organisms n scarlet fever and not in other forms of otitis media?"

As further evidence of the causal relation of these organisms to this condition, she relates the results of her serological work. The gist of this work was as follows: All pseudo-diphtheritic cases showed a very markedly lowered opsonic index to this bacillus. Normal persons and those suffering from streptococcic otitis media did not show a lowered opsonic index to the pseudo-diphtheria bacillus. By injections of dead cultures of homologous strains the patient's opsonin for that strain could be raised. No ill effects followed such injections, but an apparent improvement resulted in several cases. The homologous opsonin was specific. The opsonic status could be correlated with the clinical symptoms, and the clinical condition could be predicted by a change in the index.

Ruediger (1903) found a pseudo-diphtheria bacillus in enormous numbers in seven cases of scarlatina. Agglutination reactions showed the organisms to be identical. They were highly pathogenic to guinea-pigs and produced a soluble toxin which was not neutralized by diphtheria antitoxin. This is one of the organisms also found by Hamilton (above) to be pathogenic.

Cave (1912), quoted by Fox, is firmly of the opinion that *B. Hoffmannii* gives rise to unilateral tonsillitis. The disease runs a course of one to three weeks, is associated with much irritability and a fever of 104°. The Klebs-Loeffler bacillus was absent, while *B. Hoffmannii* was found in practically pure culture. Although there was no reaction on inoculation in guinea-pigs, he did not consider that this fact disproved his contention. Hewlett, Knight, Priestly, Richmond and Salter (ibid.) substantiate the view of Cave. Beyer (1898) reports a case of pharyngitis and tracheitis due to an avirulent Klebs-Loeffler bacillus.

Spronck (1896) produced marked edema and loss of weight by injection of two diphtheroid strains into guinea-pigs. The organisms were recovered from cases of angina, from which fact he concludes that diphtheroids are the cause of certain anginas.

De Witt (1912) reports a case of suddenly-developing tonsillitis with high fever. The tonsils were so large as to almost occlude the fauces. Diphtheria antitoxin proved useless. The disease ran a septic course and in one week the patient developed

ulcerous vaginitis, and a purulent discharge from both ears. Recovery took place in six weeks, and an organism was isolated in pure culture from all lesions. It was pathogenic to guinea pigs, causing local abscess, peritoneal effusion and death. A forty-eight-hour filtered broth culture killed a pig in forty-eight hours. The blood of the patient agglutinated the organism in dilution of 1–100. De Witt believes this to be a case of diphtheroid infection, and her data are very convincing.

Eyre (1896) recovered *B. xerosis* from fifteen cases of chalazion and reproduced the condition in the eyes of animals. Davis (1898) reports twelve cases of post-scarlatinal suppurative otitis media in which was found a short diphtheroid. In ten of the cases the same organism was found in the throat. One case had a membrane with no Klebs-Loeffler bacilli. The organism killed guinea-pigs in doses of 2 cc. in twenty-four hours, with general serous peritonitis, marked congestion of the organs and septicemia. Williams (1898) also isolated a similar organism from a person who had diphtheria six weeks before. It produced sepsis in guinea-pigs.

Lungs and pleura

Rosenow (1914b) reports a case of broncho-pneumonia followed by arthritis and endocarditis. From the blood of this patient he recovered a pure culture of a diphtheroid bacillus producing in rabbits pulmonary hemorrhage, broncho-pneumonia, multiple nonsuppurative arthritis, myocarditis and local infection of the muscle in the stomach. Klein (1903) reports the recovery of a diphtheroid obtained from the hepatized lung of a white rat. Inoculation of the organism into rats produced hepatization of the lung and hemorrhage. He demonstrated the organism in the tissues and recovered them in pure culture. Sepsis was also produced. My strain 26 was isolated by J. Howard Brown in pure culture from a lung abscess in a guinea-pig. He feels that it was the causative organism in this case. In another part of this paper I have demonstrated a diphtheroid to be the cause of a very curious interstitial pneumonia. (strain 1) (Journal of Bacteriology, **2**, 53.)

Gastrointestinal system, liver and pancreas

Rosenow reports the isolation of a diphtheroid from acute ulcers in the stomach, in a case of acute fatal arthritis following extraction of an abscessed tooth in a patient of Dr. Billings. There were other pathological processes in the same patient from which the organism was isolated. It seemed to be the cause of the entire condition.

Hektoen (1901) has conclusively shown that a diphtheroid may produce cirrhosis of the liver. His organism was isolated from a case of blastomycotic dermatitis of the hand. In guinea-pigs it produced a cutaneous ulcer at the site of injection, and the animal died of emaciation in three to five weeks. It constantly produced necrosis and cirrhosis of the liver, and part of the time sepsis. The changes were confined to the liver in the guinea-pig. The major pathological processes were: perilobular connective tissue, proliferation with hyperplasia of the biliary passages and small areas of focal necrosis. In these lesions Hektoen demonstrated the causative organism by stain and by culture. Similar results were produced with filtered cultures, although they rarely caused death. Diphtheria antitoxin gave no protection.

It is noteworthy that subcutaneous or intravenous doses did not affect the liver in rabbits, although injection into the anterior chamber of the eye caused the typical liver lesions to develop. To a lesser extent it was possible to produce the hepatic changes in dogs, but not in the grey mouse or white rat.

Rosenow's (1915b) erythema nodosum strain has produced acute hemorrhagic pancreatitis in experimental animals.

Genito-urinary system

G. F. Dick and G. R. Dick (1915) report a series of twenty cases of acute and chronic nephritis in which they constantly found a small, pleomorphic, Gram-negative, anaerobic diphtheroid and a streptococcus. They killed laboratory animals with both of these organisms, producing albuminuria and acute

degenerative changes in the renal parenchyma. Doses of the diphtheroid as low as 2,500,000 produced headache and nausea of the same character in the patients as that from which they had been suffering. An increase of the albumin was also observed.

Francioni, quoted by Fox (1915a), describes a case of hemoglobinuria, cyanosis and jaundice without fever in which the pseudo-diphtheria bacillus was thought to be a causative factor. It was present in the blood during life, and in large numbers in all the organs after death.

Townsend (1905) reports a case of pseudo-membranous and fibrosing cystitis caused by the pseudo-diphtheria bacillus. Rosenow (1915c) isolated a diphtheroid organism from the urine in a case of ulcerative cystitis. It produced septicemia in guinea-pigs. No lesions were described. The patient's serum agglutinated specifically in high dilutions. There was no agglutination of the colon bacillus with which the organism was associated. He considers that the organism has a causative relation on account of:

"1. Its constant presence in the urine and its predominance in the smears from the scrapings.

"2. The specific and peculiar behavior of the blood toward this bacillus. The patient showed marked improvement following an injection of the dead bacilli.

"3. The high agglutinin power of the serum for this bacillus and its absence for the associated colon bacillus."

Strain 2 of my series is an anaerobic, Gram-positive, fusiform diphtheroid isolated from the blood and urine of a case of parenchymatous nephritis. Small intravenous injections produced marked albuminuria and loss of weight in rabbits. The animals finally succumbed. The evidence for the relation of this organism to the condition was therefore strong.

Teacher (1915) has isolated pure cultures of *B. flavidus* from the uterus of guinea-pigs which were the victims of an outbreak of infectious abortion. Experimentally, he has repeatedly reproduced this pnenomenon in guinea-pigs with this bacillus. He shows that the bacilli have a definite seat of election, which is the cavity of the yolk sac. The organisms are recovered from

purulent areas at the sites of the placental attachments. It is quite noteworthy that the guinea-pigs show no apparent clinical results of the injection.

Skin

Rosenow (1915b) has convincingly shown that erythema nodosum is caused by a diphtheroid. He isolated the causative organism from carious teeth, tonsils, blood, throat ulcers and the erythematous nodes of the patients, and was able to reproduce the specific lesions of this disease in dogs, rabbits and guinea-pigs. He used a large number of animals, and recovered the organisms from the reproduced lesions, as well as demonstrating them microscopically therein. He obtained the same results in eight clinical cases of this condition.

Dean, quoted by Graham-Smith (1908), recovered diphtheroid bacilli from a leprosy-like disease in rats, and found that the organism was mildly pathogenic for other rats.

Meninges and brain

Warnecke (1900) found diphtheroids in pure culture in a case of otitis media. Apparently from this condition meningitis and metastatic abscess developed, and from both situations the organism was recovered.

Heart

Howard, quoted by Hamilton (1904), has described an interesting case of ulcerative endocarditis without diphtheria. He recovered pure cultures of the organism from the heart valves, spleen and kidneys. The organism was non-pathogenic for guinea-pigs. In the light of the relation which certain members of this group bear to the streptococci, the significance of these findings becomes more pertinent.

Bones and joints

Rosenow (1914b) cites a case of a duodenal ulcer which developed a septic fever two weeks following operation. In

conjunction with systematically placed nodules along the subcutaneous vein of the forearm, the patient was afflicted with a very severe pain in the femur. Pus from a tonsil yielded a diphtheroid bacillus and a streptococcus. The blood yielded a diphtheroid bacillus in pure culture. A vaccine made from the latter organism caused a disappearance of all symptoms.

I have isolated a diphtheroid bacillus in pure culture from an acute swelling in both popliteal spaces in a case of chronic arthritis. A culture was obtained also from the blood. Agglutinin reactions were positive. Vaccines made from the organism cleared up the acute condition and very markedly relieved the chronic arthritis. Voigt[2] has isolated a diphtheroid in pure culture from the urethra in a case of gonorrhoeal rheumatism. This organism had much the same characteristics as my streptococcoid strain 1, and gave positive agglutination reactions with the patient's serum. An autogenous vaccine caused a marked improvement in the patient's condition, which was of significance inasmuch as he had failed to respond to a gonococcus vaccine. Experimentally the erythema nodosum diphtheroid and strain 1 of this series constantly produced arthritis.

LYMPHATIC SYSTEM

Bunting is the most prolific contributor to the diseases of this system. He has isolated a very pleomorphic, antiformin-fast, non-acid-fast, Gram-positive diphtheroid from the lymph glands of several cases of Hodgkin's disease. He considers this organism identical with the one described by Frankel and Much (1910) as the probable cause of this condition. He describes in detail the various stages of the tissue lesions which he has produced experimentally. They are typical of the pathology of this disease as described by Reed, Longcope and others. He has also produced in monkeys the blood picture which he considers characteristic of the condition in the human subject. Furthermore, this blood picture has been produced in man by the injection of the killed *B. Hodgkini*. He has also produced in mon-

[2] Personal communication.

keys the acute form of the disease which occasionally occurs in man. At the present time his experimental results remain unconfirmed.

Rosenow (1915a), Rhea and Falconer (1915), Kolmer, quoted by Fox (1915b), Klotz and Holman,[3] Torrey (1916), Fox (1915b), Wade and Harris (1915) and many others have isolated diphtheroid organisms from Hodgkin's lymph glands as well as from other locations, but they have never been able to show that they were pathogenic.

Bunting also claims to have isolated similar diphtheroids from Banti's disease and reproduced the condition in dogs. From pseudo-leukemia, lymphosarcoma and acute and chronic leukemia a diphtheroid similar to *B. Hodgkini* has been isolated by Bunting, who takes the position that these various conditions are different expressions of the toxic action resulting from various strains of the same diphtheroid subgroup.

Steele (1914) has described an antiformin-fast bacillus from the spleen in a case of acute leukemia which he thinks is identical with *B. Hodgkini*. Simon and Judd (1915) have confirmed Steele's observations. Their strain does not ferment glucose, lactose, sucrose, mannite or inulin. Steele's is negative on glucose or lactose, which seem to be the only sugars tested.

Hall and Stone (1916) have identified the bacillus of Preiz-Nocard in caseous lymphadenitis of sheep, and in the abscesses of eleven horses and sheep. It was found for the most part in pure culture, and they believe they have established it as the cause of the condition. It was pathogenic to most of the lower animals. The same organism has been demonstrated as the cause of ulcerative equine lymphangitis by Preiz-Nocard. The organism produces orchitis and generalized suppuration throughout the lymphatic system in guinea-pigs. It is a typical member of the *B. flavidus* subgroup.

I have studied Bunting's Banti and chronic leukemia strains, which are number 43 and 44 of my series. Although resembling his *B. Hodgkini* very closely morphologically and culturally,

[3] Personal communication.

they are totally opposed as far as the sugar reactions are concerned. Neither of them ferment any of the ten sugars tested, which compares with Simon and Judd's results. They resemble *B. Hoffmannii* very closely in this respect. Bunting's pseudoleukemia strain which I also have studied can be said to be practically identical with his *B. Hodgkini*, differing only in its fermentation of inulin and salicin (strain 45).

VIRULENCE

It is a very common occurence for diphtheroid bacilli to lose their virulence rather quickly after they have been removed from a favorable to an unfavorable environment. Davis (1898) and Williams (1898) to whose experiments I have referred already report that their organism lost its virulence very quickly, so that 11 cc. was the minimal lethal dose. When first isolated 2 cc. of the broth culture killed promptly in twenty-four hours. Hektoen's (1901) bacillus which produced hepatic cirrhosis in guinea pigs "lost its virulence very soon, so that the experiments could not be continued." Hamilton (1904) has had the same experience with several of her pathogenic strains.

My strain 2 was an example of this change. It was isolated as a strict anaerobe. I was able to get it to grow aerobically by using various oxygen pressures and gradually adapting it to an aerobic environment. When this had been accomplished it was no longer pathogenic for rabbits, even in very large doses. The anaerobic culture which had never been so changed, still retains its pathogenicity. This phenomenon naturally suggests the idea that the virulence may have been recently acquired.

SYMBIOSIS

The patient from whom I isolated strain 2 could trace his clinical condition directly to an untreated wound in the foot caused by a rusty nail. A continuous fever followed for six months at the end of which time he had a severe parenchymatous nephritis. This strain was isolated from the blood in pure culture, but in the urine it was mixed with a streptococcus.

It was not unlikely that the streptococcus and the diphtheroid were introduced at the same time. Dick (1915) in his series of nephritic cases finds the diphtheroid usually associated with a streptococcus. Hektoen's pathogenic diphtheroid was isolated from a case of blastomycotic dermatitis of the hand. Hamilton's pathogenic diphtheroids have been found chiefly in scarlet fever throats, and she suggests a symbiotic relationship to account for their pathogenicity. I have obtained some very suggestive experimental evidence along this line, but it is too incomplete to consider as yet. An apriori argument for such a contention lies in the wonderful lability of the group as a whole. Its ability to take on various forms I have already sufficiently emphasized. The same race may vary in its staining properties within wide limits. This I have noted in relation to Gram's stain, to acid-fast stains, and to the metachromatism with Loeffler's. Culturally it is one of the most adaptive of organisms, which may explain its characteristic viability. It is possible that a group with such a labile chemical base would be quite susceptible to symbiotic changes.

One of the most ideal places for such changes to be developed lies in the foci of infection in the crypts of the tonsils, pus pockets in the teeth, etc. It is Rosenow's idea that streptococci acquire their elective affinities in this way. It is noteworthy that many of his cases of erythema nodosum have yielded the infecting organism from one or both of these locations. Clinically this condition is a sequel of streptococcic tonsillitis, and a possible symbiosis is suggested. Bunting (1914) traces the source of infection of one of his Hodgkin's cases to an otitis media of some duration. Again, in a primary inguinal case the patient gave a history of a sharp attack of cystitis preceding glandular involvement. Infection through the gastrointestinal mucosa has been shown where Hodgkin's disease is confined to the lymphoid tissue of that tract and the mesenteric glands. In this connection, Hektoen (1901) had an interesting experience with his strain. The organism failed to produce the characteristic hepatic cirrhosis in rabbits that obtained in guinea pigs, unless it was introduced into the anterior chamber of the eye. Subcutaneous and intravenous doses did not affect the liver.

These facts lead very logically to the consideration of the validity of the guinea-pig test as usually carried out, viz., as an infallible criterion of virulence for the diphtheroid group. In addition to what already has been adduced, there is much more information that might be focused on this point. Cave, Priestly, Richmond and Salter, Prochaska, Hewlett and Knight, Fox and others have attributed to *B. Hoffmannii* a causal relationship to a benign unilateral tonsillitis with membrane formation. The organism is usually found in pure culture, and some of these authors do not hesitate to say that the failure of the guinea-pig to die is not conclusive negative evidence, when so much positive evidence points in the other direction. Hamilton (1904) shows that four strains of diphtheroids are non-pathogenic for man and yet are pathogenic for guinea-pigs. This illustrates converse evidence. De Witt (1912) proved that a diphtheroid was the cause of a septic fever in the human being, but notes that the organism was not pathogenic for guinea-pigs, especially after the first generation. In Howard's famous case (quoted by Hamilton) a diphtheria-like organism was isolated in pure culture from the heart valves, spleen, liver and blood, but it was non-pathogenic for guinea-pigs. Klein's (1903) organism was proven to have caused hepatization in the lung of white rats, yet was either non-pathogenic for guinea-pigs or caused only a local abscess.

The admonition of Rosenow in this regard can well be called to mind; this is to inject an animal, watch it carefully, kill it in a reasonable time and *look* for lesions. Teacher and Burton (1915) discovered an epidemic of infectious abortion among their guinea-pigs and showed that the strain on reinjection into a suitable animal produced placental separation without a detectable symptom on the part of the animal. Bunting was able to get a "take" only with one of his Hodgkin's strains by multiple injections into one monkey until local abscess was produced; by transferring the contents of this abscess directly to the second monkey, he was able to kill it with symptoms of sepsis.

DISCUSSION AND SUMMARY

It would seem that there is plenty of evidence for pathogenicity on the part of the diphtheroid group—at least it is not limited to a mere handful of cases. The virulent strains quickly lose this quality, which suggests that it has been of recent acquisition. Since this type of infection so often apparently has its origin in a focus, it would seem logical that the virulence may have been acquired in that location. This evidence, coupled with the fact that the group as a whole occupies a borderline position pathologically, leads to the idea of "sub-infection" introduced by Adami and others. Sub-infections are fundamentally conditional infections to a much greater degree than is the case with the ordinary virulent infection.

It would seem logical to consider an individual on the "border line" between health and disease before he becomes susceptible to a subinfection. In such a case the validity of the guinea-pig test as usually employed would not have its ordinary significance. One would not usually expect a healthy guinea-pig to be affected by an organism which only proved pathogenic for the human species when the resistance of the latter had been impaired. It would seem probable that our attempt to infect experimentally does not approximate human conditions closely enough, and that this is the reason why we fail. Our working margin for successful infection with diphtheroids might be compared to the rapid loss of "elective affinity" of Rosenow's streptococci on artificial cultivation. His work suggests that there are many low-grade infections in man, which insidiously give rise to a very definite pathology; these changes may, however, go unnoticed until the infection either culminates critically for the patient, or what is more important from our standpoint, prepares him for a more severe infection. The acute and therefore more noticeable character of the latter may cause us to ignore the insidious antecedent changes (sub-infections) which made the acute attack possible.

The demonstration of a biological link between the diphtheroid and streptococcic groups would seem to entitle the former

to more consideration from the standpoint of pathogenicity than it has received in the past. It suggests a plea for a better interpretation of these organisms. Their discovery in certain locations may have the same meaning as the discovery of streptococci of moderate virulence. A concrete instance will illustrate my point of view.

Some time ago I had occasion to study a case of severe arthritis which had a very plain gonorrhoeal history, and clinically was a case of this sort. The patient responded to a diagnostic dose of gonococci, but the vaccine had no curative effect. Streptococcic vaccines improved the condition markedly. At this juncture the patient left the hospital to spend the week-end at a friend's home. She inadvertently spent too much time on her feet, and returned with a boggy swelling in each popliteal space about the size of a hen's egg. Puncture of each tumor under aseptic conditions yielded a pure culture of a very pleomorphic diphtheroid bacillus which fermented a wide range of sugars. Intravenous injections into rabbits developed, among other things, a non-suppurative arthritis which did not kill the animal. Subcutaneous injections in guinea-pigs caused only a transient edema at the site of injection. Autogenous vaccines gave beneficial results in the case.

I wish also to refer to the case of Voigt's (already cited), which he worked out in my laboratory. Strain 41 of this series came from the urethra of that case, and the sugar reactions will show that it belongs to the *B. enzymicus* sub-group.

The studies of Hine (1913) show that urethral diphtheroids characteristically have a wide range of fermentation. Ohlmacher years ago thought that he had beneficial results from incorporating them in his vaccines for chronic gonorrhoea. Strains 13 and 14 of my series came from a case of acute gonorrhoea, and strain 14 when given to young rabbits intravenously caused myositis, thrombosis, sepsis and death.

Further evidence bearing on this point may be derived by consulting the range of sugar fermentations of the diphtheroids isolated by Fox (1915b) from glands draining arthritic joints. Taken collectively they have a much wider fermentative range

than the other strains considered collectively. These collations are at least suggestive, and point out a direction for clinical research which may be of benefit.

Since Bunting's and my strain of *B. Hodgkini* belong to the same group and gave cross complement-fixations with the strain "Lewis streptococcus," more serious consideration of them may be merited. Lymph-gland studies show that a great diversity of diphtheroids inhabit these structures. For this reason much confusion has arisen regarding the identity of *B. Hodgkini*. Curiously enough there has been no adequate description of this organism. The sugar reactions which go a very long way toward defining it, have been omitted from nearly all descriptions. I have shown that strains 37, 38, 39, and 40 of Bunting's give uniform sugar reactions.

I have studied some of the Rosenow-Gaarde cultures, and most of them yielded at least two types of diphtheroids when plated out. The predominating type was the saprophytic *B. Hoagii* or some of its variants. Usually a member of the *B. enzymicus* group could be separated also, but this was not always the case. This experience corresponds to that of Torrey, Bloomfield, Fox and others who have studied the glandular diphtheroids. This fact may help to account for the negative animal experiments reported.

Four of these "Rosenow-Gaarde" cultures were kindly sent me by Dr. J. J. Moore from Dr. D. J. Davis's laboratory Inasmuch as lymph gland cultures yield mixed types of this organism it would seem necessary to identify *B. Hodgkini* in such mixtures. This point is interesting in connection with Moore's recent paper on "Immunologic Studies in Hodgkin's Disease" (1916). He prepares a polyvalent antiserum with 16 strains of diphtheroids isolated by Drs. Rosenow and Gaarde from Hodgkin's glands. Although this serum fixes complement with the homologous antigen in doses of 0.0005 cc., there is no fixation with the sera of ten individuals having Hodgkin's disease. If a generous portion of the organisms used in this work did not belong to the same sub-group as those with which Bunting claims to have gotten his results, the work of the two observers would be difficult to compare.

The fact that little or no serological evidence has been brought forward is an argument against the current etiological theory. Torrey makes a discouraging observation regarding any positive evidence, inasmuch as he obtains an anaerobe (*B. lymphophilis*) from Hodgkin's glands which he says reacted positively to lymphosarcoma and Hodgkin's disease to which it bore no evident relation! The great variation in colony formation possessed by a single strain gives this characteristic little differential value. The pleomorphism on which so much stress has been laid must always be correlated with the more differential biologic features.

CONCLUSIONS

1. It would appear that the sum total of evidence shows that the diphtheroid group is one of great diversity and lability, and that it is more or less related to several other groups of organisms. Prominent among these is the acid-fast group, represented by *B. tuberculosis;* the streptococcus group, represented by some of its non-hemolytic types; *B. proteus* and the enterococcus. Symbiotic relations between these groups and the diphtheroids is more than a possibility, although as yet we have little definite knowledge on this phase of the subject.

2. The group may be divided into at least seven sub-groups, viz.; *B. Hoagii*, *B. diphtheroides liquefaciens*, *B. xerosis*, *B. Hoffmannii*, *B. enzymicus*, *B. flavidus* and *B. Ruedigeri*. Among the first four are found the greater number of saprophites, while the last three subgroups yield the larger number of examples of pathogenicity. *However, any of the subgroups may become pathogenic under favorable conditions.*

3. The sugar reactions form a fairly reliable criterion for the delimitation of these groups, when a sufficient number of sugars are employed and the various technical and other limitations regarding their utility in this respect are observed. The cultural, morphological and immunological characteristics can be successfully correlated with a grouping made on this basis. The most decisive group-differentiation resulted from the use of the complement-fixation reaction.

4. The recognition and delimitation of a subgroup related to the streptococci, *B. enzymicus*, may lead to a more serious regard for the pathogenicity of certain members of the group, and at least should demand a more careful study and interpretation of diphtheroids; *B. enzymicus* may be found anywhere in the body, especially in the blood, joints and upper respiratory tract.

5. The guinea-pig test as usually employed for the determination of the virulence of the Klebs-Loeffler bacillus is by no means infallible when similarly employed with the diphtheria-like bacilli. Rabbits may prove to be better test animals, as they are more susceptible, especially with the intravenous method of injection. However, when this class of organisms is not pathogenic for laboratory animals, the inference is not to be conclusively drawn that they are lacking in pathogenic importance for man. The conditions determining their invasion of the latter species can only be imperfectly paralleled when healthy representatives of another species are employed.

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